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Glycyrrhetic acid-loaded microparticles: liver-specific delivery and therapeutic potential against carbon tetrachloride-induced hepatitis

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Abstract

The microparticles (MPs) of an anti-hepatotoxic drug, glycyrrhetic acid (GLA), were prepared using poly(DL-lactic acid-co-glycolic acid) as a drug carrier, and their in-vitro properties, biodistribution and therapeutic effects were investigated. The MPs showed a particle diameter distribution of 1.0–1.4 μm and a drug content of approximately 10% (w/w). In the in-vitro release in a mixture of methanol and phosphate-buffered saline pH 7.4 (3:7, v/v), slow release was observed after an initial burst release of approximately 30% (w/w). After i.v. administration of MPs in normal mice, GLA was mainly distributed to the liver. After i.v. administration in normal mice, the MPs maintained a much higher liver concentration than did GLA solution, and the plasma concentration also tended to be higher for MPs than for GLA solution. As to therapeutic effect, the liver was damaged by repeated injection of carbon tetrachloride (CCl_4) in mice every 48 h, and the drugs were administered intravenously as a single dose 3 h after the first injection of CCl_4. At 10 mg GLA eq. kg^{-1}, the MPs significantly suppressed the plasma level of glutamic pyruvic transaminase for at least 141 h after administration, while GLA solution did not become significantly effective within 45 h post-administration. MPs are suggested as a possible useful system to prolong the therapeutic effect of GLA.

Introduction

Microparticulate dosage forms are widely used for controlled release and/or drug targeting. Poly(DL-lactic acid) (PLA) and poly(DL-lactic acid-co-glycolic acid) (PLGA) have been used as drug carriers in this field because of their biocompatibility and biodegradability (Gilding & Reed 1979; Wakiyama et al 1981; Juni et al 1985a,b; Ogawa et al 1988a,b; Heya et al 1991; Birnbaum et al 2000). Their drug release is affected by the physicochemical properties of the drug, the molecular weight of the polymer and its lactic acid:glycolic acid ratio (Ogawa et al 1988a,b; Heya et al 1991), and some additives can modify the release properties (Juni et al 1985b; Ogawa et al 1988a). In the present study, PLGA with the above characteristics was applied as a drug carrier to prepare microparticles loaded with an anti-hepatotoxic agent, 18β-glycyrrhetic acid (GLA) (Ichikawa et al 1986; Ishida et al 1989; Takeda et al 1996).

GLA is an aglycone and an active metabolite of glycyrrhizin (GLZ), and shows various therapeutic effects, such as anti-inflammatory, anti-hepatotoxic and interferon-inducing actions (Ichikawa et al 1986; Ishida et al 1989; Higuchi et al 1992; Takeda et al 1996). GLA is effective against chronic hepatitis but is also related to the side-effect aldosteronism. Since only GLA appears in the blood circulation after oral administration of GLZ, GLA is considered to play an important role in the biological action of oral administration of GLZ (Oketani et al 1985, Ishida et al 1989). Many pharmacokinetic studies on GLZ and GLA have analysed their behaviour in the body; prolonged supply of GLA can make the effect higher and longer but also may cause side-effects such as aldosteronism. However, so far very few studies have reported on systems for the controlled release and/or targeting of GLA. In the present study, the microparticulate dosage form, which can control GLA release and localize it to the liver, was prepared and examined for in-vivo therapeutic potential. PLGA was used as a drug carrier because of its biocompatibility, biodegradability, potential to control drug release, etc., as stated

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above. The microparticles obtained were characterized in vitro for size and drug release, and evaluated in vivo by examining tissue distribution and therapeutic effect against carbon tetrachloride (CCl_4)-induced liver injury using mice.

Materials and Methods

Materials

PLGA with a molecular weight of 2×10^4 and a lactic acid unit:glycolic acid unit ratio of 3:1 (mol/mol) was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). GLA was obtained from Sigma Chemical Co. (St Louis, USA). All other chemicals were of reagent grade.

Animals

Male ddY mice (7 weeks old) weighing 30–35 g were purchased from Tokyo Laboratory Animals Science Co. Ltd (Tokyo, Japan). The animals were kept on the breeding diet MF (Oriental Yeast, Japan) with water ad libitum at room temperature maintained at $23 \pm 1\,^{\circ}C$ and a relative humidity of $60 \pm 5\%$. The experimental protocol was approved by the Committee on Animal Research of Hoshi University, Tokyo, Japan, and the animal experiments were performed in compliance with Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, Japan.

Preparation of MPs

PLGA microparticles containing GLA (MPs) were prepared by a solvent evaporation method. PLGA (100 mg) and GLA (100 mg) were dissolved in 10 mL of dichloromethane, and the solution was put quickly drop-wise into 200 mL of 1% (w/v) gelatin aqueous solution that was sonicated at 45 kHz (100 W) with stirring at 800 rpm. The sonication with stirring was continued for 10 min. While the obtained emulsion was stirred at 500 rpm under reduced pressure using a vacuum pump, the temperature of the mixture was raised gradually from room temperature to $35\,^{\circ}C$ over 1 h. The mixture was then filtered using a glass filter (SIBATA P160), the filtrate was centrifuged at 3000 rpm for 20 min, and the precipitate was obtained by discarding the supernatant. The precipitate was dispersed in 120 mL of a mixture of methanol and phosphate-buffered saline pH 7.4 (PBS) (3:7, v/v), shaken at 100 strokes per min at $37\,^{\circ}C$ for 10 min, centrifuged at 3000 rpm for 20 min and the supernatant discarded. This washing was repeated once more. The final precipitate was suspended in a small volume of water and lyophilized to obtain the MP powder.

Particle size and drug content

The MPs were suspended in water and the particle diameters were measured by dynamic light scattering using a laser light-scattering instrument ELS-800 (Otsuka Electronic Co. Ltd, Japan), in which analysis was based on the photon correlation method. The drug content was determined as follows. A specified amount of MPs was dissolved in dichloromethane and PLGA alone was dissolved in dichloromethane at a similar concentration (w/w) as a blank. These samples were measured spectrophotometrically at 249 nm. The drug content of the MPs was determined from the net absorbance calculated by subtraction of the blank sample absorbance from the tested sample absorbance.

In-vitro drug release

The in-vitro drug-release test was performed using a mixture of methanol and PBS (3:7, v/v) as a release medium. The MPs (4 mg) were put into 40 mL of the release medium, and suspended uniformly by sonication at 45 kHz for 10 s. The MP suspension was divided into glass test-tubes at an equivalent volume of 4 mL. All the test-tubes containing 4 mL of MP suspension of equivalent MP concentration were set in a water bath warmed at $37\,^{\circ}C$ at the same time, and incubated at 60 strokes per min at $37\,^{\circ}C$. After that, at a predetermined time point, one of the incubated test-tubes was removed and immediately centrifuged at 3000 rpm for 10 min. The supernatant was filtered with a membrane filter (0.45 μm pore size) and the filtrate was measured spectrophotometrically at 249 nm to determine the amount of released GLA. This operation was performed for each incubated test-tube at predetermined incubation time points of 10 min, 1, 2, 4, 6 and 24 h. The incubation study was performed in triplicate.

Tissue distribution and excretion after i.v. administration of MPs

The MPs were suspended in saline and injected into normal mice at a dose of 10 mg GLA eq. kg^{-1} (10 mL kg^{-1}) via a tail vein ($n = 4$). The mice were killed by dislocation of the cervical vertebrae at 1, 12 and 24 h after administration, blood (1 mL) was taken from the heart using a heparinized syringe and the liver, kidney, spleen, lung and heart were excised. Plasma was obtained by centrifugation of the blood at 3000 rpm for 10 min. Each tissue was washed briefly with saline and wiped briefly with a filter paper. Saline was added at the same volume as the tissue volume, then the mixture was homogenized using a glass homogenizer with a Teflon pestle. To the plasma or tissue homogenate (0.1 mL), 1 mL of 0.1 M acetate buffer, pH 5.0, and 5 mL of dichloromethane were added, and the mixture was shaken vigorously for 1 min with a vortex mixer. After centrifugation of the mixture, 4 mL of the organic layer was taken, and the organic solvent was evaporated at $40\,^{\circ}C$. The residue was dissolved in 50 μL of methanol, and 20 μL was injected on an HPLC column to determine the concentration of GLA. The concentration was corrected using the recovery ratio described below. The recovery ratio from plasma or tissues was determined as follows. A specified amount of MPs was added to fresh plasma or two-fold diluted tissue homogenate, treated and

analysed in the same manner as the test sample. The ratio of the amount of GLA recovered in organic solvent to the added amount was used as the recovery ratio.

Urinary and fecal excretions were examined independently of tissue distribution studies (n = 3). Each normal mouse was bred separately in a cage for metabolite analysis. An MP suspension in saline was injected into the mice at a dose of 10 mg GLA eq. kg^{-1} (10 mL kg^{-1}) via a tail vein. At 24 h post-administration, the urine collected in the cage was mixed with the urine taken from the bladder of the mouse after it had been killed by dislocation of the cervical vertebrae, and the mixed urine was measured for volume. The feces were collected in the cage for 24 h, diluted four-fold with saline and homogenized using a glass homogenizer with a Teflon pestle. The urine and fecal homogenates were treated and analysed in the same manner as plasma or tissue homogenate. The concentration was corrected with the recovery ratio. The recovery ratio was also determined in the same manner as that in plasma or tissue homogenate.

GLA concentrations in plasma and liver after i.v. administration of MPs and GLA solution

The distribution of GLA in the plasma and liver was compared between MPs and GLA solution. This experiment was performed independently of the above tissue distribution study. MPs were administered intravenously at a dose of 10 mg GLA eq. kg^{-1} (10 mL kg^{-1}) in the same manner as above, and the plasma and liver concentrations of GLA at 1, 12 and 24 h after administration were analysed in the same way as above (n = 3). As for the GLA solution, GLA was dissolved in 2% (w/v) Tween 20 aqueous solution, and the solution (approximately 0.3 mL) was injected via a tail vein into normal mice at a dose of 10 mg kg^{-1} (n = 3). The GLA concentrations in plasma and liver were examined in the same manner as those in the MPs. The concentration was corrected by the recovery ratio as for the MPs.

Therapeutic effect against CCl_4-induced liver injury

The mice were injected intraperitoneally with a 25% (v/v) CCl_4 solution in olive oil at a dose of 4 mL kg^{-1}. CCl_4 was repeatedly injected in the same manner at 48 and 96 h after the first CCl_4 injection. At 3 h after the first CCl_4 injection, an MP suspension in saline was administered to the tail vein at a single dose of 5 and 10 mg GLA eq. kg^{-1} (10 mL kg^{-1}), and GLA solution in 2% (w/v) Tween 20 aqueous solution was administered intravenously at a single dose of 10 mg GLA eq. kg^{-1} (10 mL kg^{-1}). At 24, 48, 72, 96, 120 and 144 h after the first CCl_4 injection, the mice were killed by dislocation of the cervical vertebrae, and blood (1 mL) was taken from the heart with a heparinized syringe. The plasma was obtained by centrifugation of the blood at 3000 rpm for 10 min. When the blood sampling and injection of CCl_4 overlapped, blood sampling was performed earlier. These schedules are shown in Figure 5A. The plasma levels of glutamic pyruvic transaminase (GPT) were determined based on the UV rate method (Karmen et al 1955) using a GPT-UV Test Wako kit (Wako Pure Chemical Industries Ltd, Japan). The therapeutic effect was evaluated from the prevention extent of the enzyme levels against the control, i.e. the prevention effect was evaluated at each time point using the following equation:

$$\text{Prevention effect } (\%) = 100 \times (\text{GPT mean level in control} - \text{GPT level in tested sample})/(\text{GPT mean level in control}) \quad (1)$$

HPLC assay

HPLC analysis was performed at room temperature using a Shimadzu LC-6AD with a UV-VIS detector (Shimadzu SPD-10AV) set at 254 nm. A Nucleosil 100-5C_{18} column (4.0 mm in inner diameter × 250 mm in length) (GL Sciences Inc., Tokyo, Japan) with a Nucleosil 100-5C_{18} guard column (4.0 mm in inner diameter × 50 mm in length) (GL Sciences Inc., Tokyo, Japan) was used for analysis. The mobile phase was a mixture of methanol, water, acetic acid and perchloric acid (87:17:0.5:0.5, v/v/v/v), and the flow rate was set at 0.8 mL min^{-1}. Twenty microlitres of the sample was injected onto the column.

Statistical analysis

Comparison of two results was performed using the unpaired *t*-test, and comparison of three or more results was executed using ANOVA followed by Bonferroni/Dunn post hoc. The difference was considered significant at $P < 0.05$.

Results

Particle characteristics

The MPs were obtained at a yield of 50–60% for PLGA. From dynamic light scattering, the MPs showed a particle diameter distribution of 1000–1400 nm with a mono-dispersed particle diameter distribution in aqueous suspension (Figure 1). The GLA content in the MPs was 9.7% (w/w). The encapsulation efficiency, defined as the ratio of observed drug content to ideal drug content, was 19%.

In-vitro drug release

The release profiles from the MPs are shown in Figure 2. An initial rapid release of 30% (w/w) was observed, and the drug was released gradually up to 56% (w/w) 24 h after the start of the release test. In the preliminary study, the microparticles obtained by washing with water (120 mL) three times (MPWs) showed a mean particle diameter of 1300 nm and a drug content of 50% (w/w). The MPWs were examined for in-vitro release under the same conditions of 0.1 mg particles mL^{-1} in a mixture of methanol and PBS (3:7, v/v). The MPWs exhibited a high initial rapid release of nearly 80% (w/w) (Figure 2).

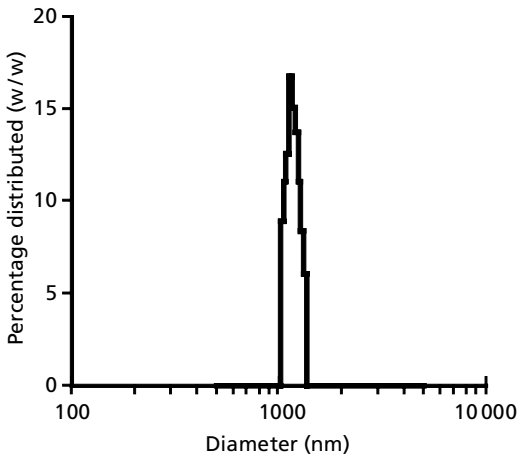

Figure 1 Particle size distribution of MPs.

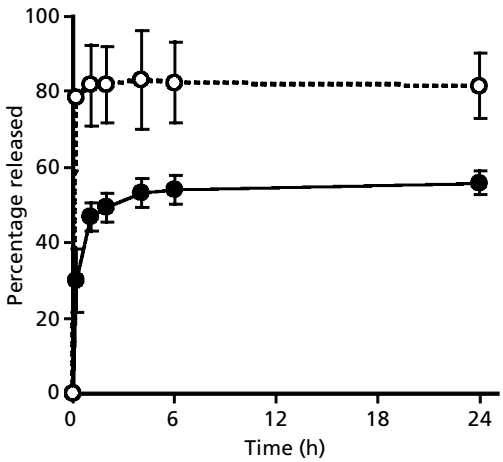

Figure 2 In-vitro release profiles of GLA from MPs. The closed and open circles represent MPs and MPWs, respectively. The results are expressed as the mean ± s.d. (n = 3).

In both MPs and MPWs, the calculated concentration corresponding to 100% release was much lower than GLA solubility (>200 $\mu g\,mL^{-1}$) in this medium.

Tissue disposition and excretion after i.v. administration of MPs

The amounts of GLA distributed in plasma and tissues are shown in Figure 3. The excretion extents in urine and feces after i.v. administration of MPs are described in Table 1. These correspond to the amounts of incorporated plus free GLA. The recovery ratios were determined for the total (incorporated plus free) GLA. The recovery ratios were more than 70% of GLA in all the tested samples, and almost the same in both incorporated and free GLA, i.e. the recovery ratios are 89, 74, 75, 80, 80, 89 and 82% for plasma, liver, spleen, heart, kidney, urine and feces, respectively. At 1 h after i.v. administration of MPs, 93% of the

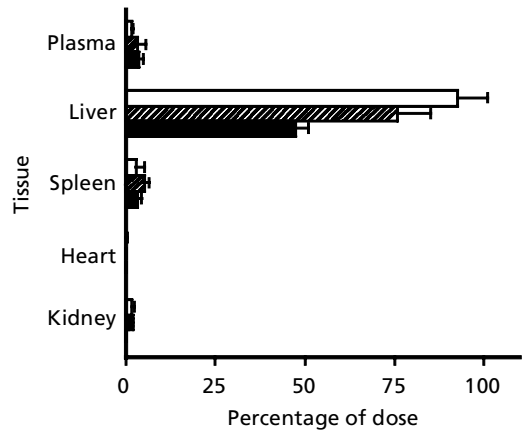

Figure 3 Biodisposition of GLA after i.v. administration of MPs at 10 mg GLA eq. kg^{-1} in normal mice. The white, hatched and black columns represent the distribution amounts of GLA (percentage of dose) at 1, 12 and 24 h after i.v. administration, respectively. The results are expressed as the mean ± s.e.m. (n = 4).

Table 1 Cumulative urinary and fecal excretion of GLA for 24 h after i.v. administration of MPs to normal mice at 10 mg GLA eq. kg^{-1}.

Excretion	Cumulative amount (% of dose)
Urine	0.43 ± 0.13
Feces	30.22 ± 1.88

Results are expressed as mean ± s.e.m. (n = 3).

dose was distributed in the liver, while the located amounts were less than 2% of the dose in the plasma, spleen and kidney. The liver GLA was eliminated gradually and reached 47% of the dose at 24 h post-administration. The distributed amounts of GLA in plasma and all other tested tissues were less than 4% during the 24 h after administration. The distribution to the lung could not be quantified clearly by the present HPLC method. The amount excreted in the urine was less than 0.5% of the dose in the 24 h after i.v. administration, and 30% of the dose was excreted in feces in the 24 h after administration.

Comparison of GLA levels in plasma and liver between MPs and GLA solution

The GLA concentration was determined in the plasma and liver after i.v. administration of MPs and GLA solution. The results are described in Figure 4. For the MPs, the plasma levels were 4 $\mu g\,mL^{-1}$ at 1 h post-administration, and increased gradually up to 10 $\mu g\,mL^{-1}$ at 24 h post-administration. On the other hand, after administration of GLA solution, the plasma concentration was eliminated slowly from 4 $\mu g\,mL^{-1}$ at 1 h post-administration to 0.6 $\mu g\,mL^{-1}$ at 24 h post-administration. However, the plasma levels were not significantly different between the MPs and GLA

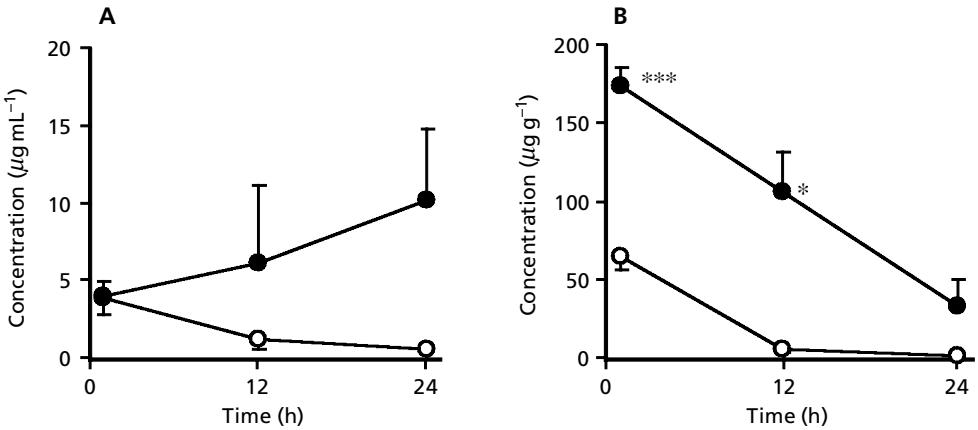

Figure 4 GLA concentration in the plasma (A) and liver (B) after i.v. administration of MPs (●) and GLA solution (○) at 10 mg GLA eq. kg^{-1} in normal mice. The results are expressed as the mean ± s.e.m. (n = 3). *$P < 0.05$, ***$P < 0.001$ as compared with GLA solution.

solution at each time point ($P > 0.05$). After administration of MPs, the liver concentration was 175 $\mu g\,g^{-1}$ at 1 h post-administration, and decreased gradually to 34 $\mu g\,g^{-1}$ at 24 h post-administration. As to GLA solution, the liver concentration was 66 $\mu g\,g^{-1}$ at 1 h post-administration, and decreased gradually to 2.5 $\mu g\,g^{-1}$ at 24 h post-administration. The MPs showed significantly higher liver concentration of GLA than GLA solution at 1 h ($P < 0.001$) and 12 h post-administration ($P < 0.05$).

Therapeutic effect against CCl_4-induced liver injury

The i.p. injection of CCl_4 caused an extremely high GPT level 24 h after injection of CCl_4, but this level recovered to a fair extent by 48 h after injection of CCl_4, as shown in Figure 5B. In this experiment, the repeated injections of CCl_4 every 48 h were performed to continue the liver damage, i.e. to maintain the high GPT levels (Figure 5A). At that time, GPT levels were kept significantly higher than the initial level except at 72 and 96 h. MPs and GLA solution were administered intravenously at a single dose 3 h after the first injection of CCl_4. At 24 h after the first injection of CCl_4, the GPT level was significantly lower at 10 mg GLA eq. kg^{-1} for both MPs and GLA solution than for the control, but not at 5 mg GLA eq. kg^{-1} for MPs, and the GPT level was significantly lower in GLA solution than in the MPs (5 and 10 mg GLA eq. kg^{-1}). For 48 h and more after the first injection of CCl_4 the GPT levels were more suppressed in the order of MPs (10 mg GLA eq. kg^{-1}) > MPs (5 mg GLA eq. kg^{-1}) > GLA solution (10 mg GLA eq. kg^{-1}). Only MPs (10 mg GLA eq. kg^{-1}) exhibited significantly lower GPT levels than the control.

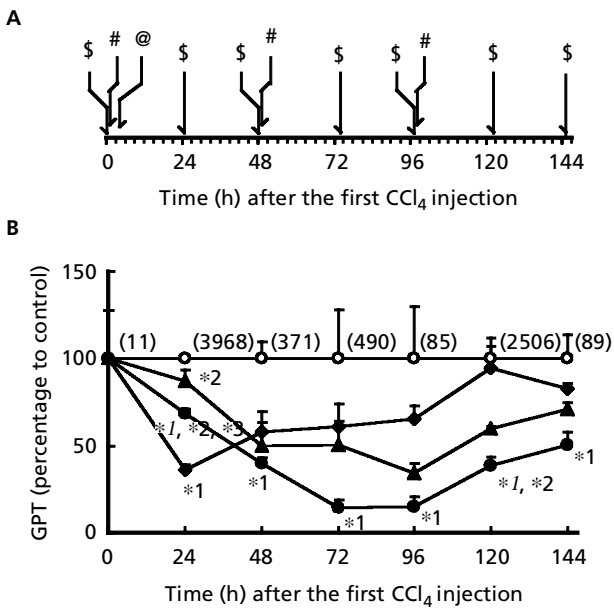

Figure 5 Animal experiment schedules (A) and therapeutic effects (B) of MPs and GLA solution against CCl_4-induced liver injury in mice. A: $, blood sampling; #, CCl_4 injection; @, drug administration. (B) The mean GPT levels of the control were normalized at 100%, indicated by open circles. The value in parentheses shows the GPT level (Karmen units) of the control at each observation time. The closed rhombuses, closed triangles and closed circles represent GLA solution (10 mg GLA eq. kg^{-1}), MPs (5 mg GLA eq. kg^{-1}) and MPs (10 mg GLA eq. kg^{-1}), respectively. The results are expressed as the mean ± s.e.m. (n = 3). Differences were significant as follows: *1 vs control, *2 vs GLA solution, *3 vs MPs (5 mg GLA eq. kg^{-1}).

Discussion

GLA is known as a very useful anti-hepatotoxic agent, and has anti-inflammatory and anti-viral potentials. Its pharmacokinetics have been examined widely for different administration routes. GLA conjugates (metabolized GLA) and GLZ are biliarily excreted, undergo hydrolysis by bacteria in the gastro-intestinal tract and are re-absorbed there (Takeda et al 1996; Ploeger et al 2001). In oral administration of GLZ at a therapeutically effective dose, the plasma level of GLZ was less than the detection

limit (10 ng mL^{-1}), but GLA was detected in plasma, suggesting that GLA is important for the therapeutic effect (Oketani et al 1985). GLA is also reported to be more effective against CCl_4-induced liver injury than GLZ in i.p. administration to mice (Higuchi et al 1992). Furthermore, GLA and GLZ are administered repeatedly for a long period for treatment of chronic hepatitis. The development of a delivery system of GLA to the liver is therefore considered very important for improving the efficacy of GLA (MPs) against hepatic injury. In the present study, the development of microparticles of GLA (MPs), showing a prolonged release and passive liver targeting, was attempted, and the MPs obtained were evaluated in vitro and in vivo.

Generally, the bidistribution of microparticles is known to be influenced by particle size, surface properties, etc. (Sugibayashi et al 1979; Kanke et al 1980; Yoshioka et al 1981; Dunn et al 1997). In particular, particle size is essentially related to tissue distribution. Microparticles of 0.3–3 μm are possibly available for passive targeting to the liver, which is based on trapping by reticuloendothelium. This particle size-dependent live passive targeting is considered as a possible useful concept against hepatic diseases. For example, such size-dependent liver passive targeting of an antitumour agent, adriamycin, was reported to be useful for a liver cancer model (Morimoto et al 1981). Since the MPs obtained in the present study exhibited a particle size of 1.0–1.4 μm (Figure 1), they were considered possibly to be adequate for passive liver targeting. Furthermore, the hydrophobicity of the matrix polymer, PLGA, might be favourable for hepatic reticuloendothelial uptake (Dunn et al 1997).

The MPs exhibited an initial burst release of 30%, then a gradual drug release, and 44% of the drug remained in the MPs 24 h after the start of the release test (Figure 2), which suggests that the MPs are available for sustained release of GLA. The MPWs showed a high drug content and adequate particle size as described above, but their initial rapid release was much greater than that of the MPs (Figure 2). The MPWs were therefore not considered to be adequate as a prolonged release dosage form, and were not used in the in-vivo study. The solubility of GLA was greater than 200 μg mL^{-1} in the mixture of methanol and PBS (3:7, v/v), but lower than 200 μg mL^{-1} in water or PBS, although each solubility was not examined exactly. On the other hand, the amount of PLGA recovered in the particles scarcely decreased after washing with the mixture of methanol and PBS (3:7, v/v) (data not shown), indicating that the PLGA matrix was hardly soluble in the mixture of methanol and PBS (3:7, v/v). These results suggest that GLA located near the surface of the MPs is washed out by washing with the mixture of methanol and PBS (3:7, v/v) without dissolving the polymer matrix. Actually, it was confirmed from the measurement of the amount of GLA washed out into the washing medium that most of the GLA related to initial burst release was removed by washing at the conditions described in the present method; at this time, the amount of PLGA recovered in the particles hardly changed (data not shown). In the in-vitro release examination, the mixture of methanol and PBS (3:7, v/v) was also used as a release medium for the following reasons. When MPs and MPWs were incubated in the same conditions of 0.1 mg particles/mL in that medium, their calculated concentrations at 100% release were considerably lower than the GLA solubility, i.e. saturation could be avoided sufficiently. Furthermore, use of this medium allowed us to measure the concentration of the released drug by simple spectrophotometry. However, a better medium may exist in the in-vitro experiments, which should be a future subject for investigation. The results of the in-vitro release showed that approximately 44% (mean value) of GLA remained in the MPs after 24 h of incubation; this residual part of GLA was considered to be retained very tightly inside the MPs. On the other hand, when the MPWs were incubated for 24 h, approximately 18% (mean value) of GLA was retained in the particles without being released, when a large deviation of several milligrams was found in the residual amount. This suggests that GLA located near the surface, corresponding to 80% (w/w) or so of the total drug, is released rapidly first, then GLA located inside is released slowly. However, for the MPWs, the calculated amount of GLA retained inside after 24 h of incubation deviated to a large extent. The large deviation of in-vitro release in MPWs also suggests that washing with water is inadequate to obtain the microparticles exhibiting a reproducible drug-release profile, namely that the drug near the surface should not be washed uniformly due to the low solubility of GLA in water. On the other hand, the MPs showed a slow-release profile with a slight deviation. This indicates that the MPs are superior as a slow controlled-release dosage form. The MPs showed a very slow release from 4 h after the start of the release test, but a release profile in a time-scale of longer than 24 h is needed to grasp the release pattern exactly from 24 h after the start of the release test. The degradation of PLGA might influence the release profile during incubation for a long time. The present release could represent the overall release features of the MPs, although a release study over a longer time-scale will also be a future subject for study as well as optimization of the release media discussed above.

At 1 h after i.v. administration of the MPs GLA was localized exclusively to the liver (Figure 3). GLA was then gradually eliminated and 47% remained at 24 h after i.v. administration. This indicates that the MPs are localized quickly by hepatic reticuloendothelial uptake. When the liver concentration of GLA was compared between MPs and GLA solution, it was much higher in the MPs than in GLA solution (Figure 4). It has been reported that GLA tends to be distributed mainly in skin and muscle and is not localized in the liver (Ishida et al 1989). The localization of GLA to the liver, which was observed to some extent 1 h after i.v. administration of GLA solution in the present study, may be due to the GLA/Tween 20 formation used for solubilization. The GLA concentration greatly decreased at 12 and 24 h after i.v. administration of GLA solution. On the other hand, the liver concentration of GLA decreased slowly after i.v. administration of MPs, which was consistent with the slow drug release from the MPs to a reasonable extent. The profiles did not appear to completely parallel each other, which was considered to be due to the difference between in-vitro and

in-vivo conditions. Furthermore, the amount of GLA distributed in the liver 24 h after i.v. administration of the MPs appears to be less in Figure 4 than in Figure 3 because one of the three mice exhibited a low liver concentration 24 h after i.v. administration in Figure 4.

The plasma concentration decreased quickly in GLA solution, but increased gradually in the MPs. This suggests that the drug released from the MPs and/or some part of the MPs taken up by the tissues, mainly in the liver, might re-circulate in the bloodstream. The lung concentration of GLA could not be determined by the present method. However, after i.v. administration of MPs, GLA localized rapidly and exclusively in the liver, i.e. more than 90% of GLA was observed in the MPs at 1 h post-administration although GLA itself did not undergo liver localization specifically, as stated above (Ishida et al 1989), indicating that entrapment of the MPs by the lung is slight. Furthermore, the MPs were excreted mainly in feces, and hardly at all in urine (Table 1), which is consistent with the report that GLA mainly undergoes biliary excretion as conjugates (GLA metabolites) and shows very slight urinary excretion (Kawakami et al 1993). Thus, the MPs exhibited an excellent liver-specific delivery of GLA, as expected. These biodistribution characteristics of MPs also suggest that PLGA microparticles of 1000–1400 nm might be useful for liver targeting of other drugs.

The i.p. injection of CCl_4 was performed at 48 h intervals to continue the liver damage for a long period. Using this treatment, the control GPT levels were much higher than the initial ones during the experiment (Figure 5). This model was therefore considered to reflect the liver injury continuing for a long period, at least up to 144 h after the first CCl_4 injection. GLA solution suppressed the GPT level best at 24 h after the first CCl_4 injection, but the prevention effect was weakened from 48 h after the first CCl_4 injection; actually, the GLA solution did not show significantly lower GPT levels than the control from 48 h after the first CCl_4 injection. The prevention effect of GLA solution appeared to a maximal extent at 24 h after exposure to CCl_4, and was not maintained for long. This was consistent with fast GLA elimination from the plasma and liver after i.v. administration of GLA solution (Figure 4). On the other hand, the prevention effect of the MPs was maintained for a long period and strengthened with increases in the dose. In particular, the MPs (10 mg GLA eq. kg^{-1}) showed a significant suppression of the GPT level for the observation period up to 144 h after the first CCl_4 injection. The percentage of prevention effect of the MPs was maximized at 72–96 h after the CCl_4 injection, and lowered more slowly. This demonstrates that MPs show a more prolonged liver protection effect than GLA solution. These features of MPs were considered to be due to prolonged release and liver localization of GLA after i.v. administration of the MPs. MPs are suggested to be a possibly useful drug delivery system for improving the prevention effect of GLA. Hence, examination of side-effects will be needed to elucidate the usefulness of MPs more clearly because the prolonged supply of GLA may lead to side-effects such as aldosteronism.

Conclusion

We have demonstrated that the present PLGA microparticles containing GLA show gradual drug release, passive liver targeting and a prolonged liver protection effect against CCl_4-induced liver injury. In i.v. administration at 10 mg GLA eq. kg^{-1}, the MPs were distributed mainly to the liver, kept the liver and plasma concentrations higher than GLA solution, and suppressed the GPT level significantly longer than GLA solution in the present liver injury model. These features were considered to be due to the prolonged drug release and high liver localization of the microparticles. The present GLA-loaded microparticles may be a possible useful drug delivery system for GLA, showing a prolonged anti-hepatotoxic effect.

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