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Research paper

Relation between dissolution profiles and toxicity of cisplatin-loaded microspheres

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Abstract

The aim of this study is to evaluate and compare the dissolution profiles of cisplatin-loaded microspheres (CDDP-MS) in vitro and in vivo, and to determine the relationship between the dissolution profiles in vitro and systemic toxicity. For this purpose, three types of CDDP-MS that release the CDDP for 1, 2 and 5 weeks without a large amount of initial release in phosphate buffered saline (pH 7.4) were prepared. The dissolution profiles of these formulations in vivo were well correlated with in vitro studies, and resulted in well-controlled plasma platinum concentration. The systemic toxicity of the CDDP-MS and CDDP dissolved in saline (CDDP-SOL) were assessed by intraperitoneal administration in mice. The maximal tolerable dose (MTD) of CDDP-SOL was 13.4 mg/kg, whereas the CDDP-MS of 1, 2 and 5-week types were 34.6, 44.2, 62.6 mg/kg, respectively. The MTD of CDDP increased proportionally when 50% of CDDP had been released from MS in vitro (MTD (mg/kg) $= 5.22 \times T_{50(\mathrm{day})} + 13.2$, $R^2 = 0.9935$). We demonstrate that the systemic toxicity of CDDP-MS can be predicted by evaluation of the dissolution rate in vitro since in vivo dissolution was correlated with the in vitro.

Keywords: Cisplatin; Microspheres; In vitro–in vivo correlation; Systemic toxicity; Dissolution profile

1. Introduction

Cisplatin (*cis*-diamminedichloroplatinum(II), CDDP) has been used successfully in cancer chemotherapy. The major side effect, nephrotoxicity, is dose limiting and occurs either acutely or after repeated treatment [1]. To reduce its nephrotoxicity while retaining the anti-tumor effect, the tumor should be selectively exposed to the drug while exposure to kidney is minimized. In order to achieve high drug concentrations selectively in the tumor, various routes of administration such as intratumoral and intraperitoneal (i.p.) have been studied [2,3]. However, these methods are often unsuccessful because CDDP administered in solution is rapidly absorbed into the systemic circulation, making it difficult to maintain a high concentration of the drug in the target region [4,5]. Investigators have therefore attempted to sustain the concentration of CDDP in the target site by means of *Drug Delivery Systems* such as disks, pellets, and microspheres (MS) [6–9]. Encapsulation of CDDP in biodegradable MS (CDDP-MS) has been carried out previously by numerous groups [8,9]. The clinical use of this preparation demonstrated a prolonged release of CDDP, however the formulation was hampered by a rapid release of CDDP initially following administration [9]. The large amount of initial-released CDDP in that preparation left the kidney at risk from high systemic exposure. To reduce this initial release, we established a new technology for the preparation of MS called as 'polymer alloys method' [10]. When applying this technology, a multi-reservoir type MS, which contains CDDP in the internal phase, can provide a long-term sustained release without large amount of initial release.

Like other anti-tumor agents, the safety range for CDDP concentration in plasma is relatively narrow. Thus, a slight change in the dissolution profile of CDDP-MS may have a profound influence on systemic toxicity. It is therefore important that the relationship between the dissolution profile and the systemic toxicity of CDDP-MS be evaluated precisely. However, only a few attempts have been made so far to evaluate the dissolution profile in vivo and systemic toxicity of CDDP-MS. Moreover, in order to anticipate the systemic toxicity of CDDP-MS from the dissolution profile in vitro, it is necessary to relate the dissolution profile of in vitro with that in in vivo.

Thus, the aims of this study are to (a) evaluate the relationship between the dissolution profiles of CDDP-MS in vitro and in vivo, (b) evaluate the relationship between the

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dissolution profiles and CDDP-MS-induced systemic toxicity and (c) investigate the effect of microencapsulation on the reduction of systemic exposure.

2. Materials and methods

2.1. Materials

CDDP was obtained from Haraeus GmbH Produktbereich Chemie (Hanau, Germany). Poly(DL-lactic acid) (PLA) and poly(DL-lactic-*co*-glycolic acid) (PLGA) were obtained from Mitsui chemicals (Tokyo, Japan). Poly(vinyl alcohol) (EG-40) was purchased from Nihon Synthetic Chemical Industries Ltd. (Tokyo, Japan). Sodium carboxymethyl cellulose (TS-1) was purchased from Nichirin chemical kogyo (Hyogo, Japan). Tween 80 was purchased from Sigma Chemical (St. Louis, USA). Saline (Otsuka normal saline) was purchased from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals were of reagent grade.

2.2. Preparation of MS

CDDP-MS was prepared according to the solvent evaporation method. In brief, PLA and PLGA were dissolved following dispersion of pulverized CDDP crystals in methylene chloride. This oil-phase was added into water containing PVA at a concentration of 0.5% (w/v) and emulsified using the Polytron homogenizer (Polytron, Kinematica Ag Littau, Switzerland). The resultant emulsion was added into water to remove the solvent. The hardened microspheres were then washed with water, filtered, and dried by lyophilization. The composition of CDDP-MS formulations is shown in Table 1. Formulations 1 and 2 were composed of PLGA and PLA at various blend ratios. In contrast, formulation 3 was composed of two PLAs having different molecular weights.

2.3. Dissolution characteristics in vitro

About 10 mg of MS was weighted out in the test tubes. The tubes were filled with 10 mL of phosphate buffered saline (PBS, 0.15 M, pH 7.4) as the test fluid. After sealing, the tubes were incubated with stirring at 25 rpm in an air chamber maintained at 37 ± 1°C. Each test tube was taken out at the predetermined day interval. The eluate, obtained by centrifuge was assayed for CDDP concentration by the reversed phase HPLC. The HPLC conditions were as follows: mobile phase consisted of 6 mmol/L 1-octanesulfonate, 6 mmol/L tetra-*n*-butylammonium hydrogen sulfate and 20 mmol/L potassium dihydrogen phosphate, adjusted to pH 5.0 with sodium hydroxide with a flow rate of 0.7 mL/min. The analytical column used was ODS 120A (4.6 × 250 mm, Tosoh Corp, Tokyo, Japan) at a constant temperature of 45°C. The spectrophotometric detector was set at 301 nm. The day when 50% of CDDP has been released from the MS in vitro (T_{50}) was calculated from regression analysis by the least squares method.

Table 1
Composition of CDDP-MS formulations

	Polymer species	Molecular weight (kDa)	Blend ratio[a]
Formulation 1	PLGA	6.7	0.4
	PLA	19	0.5
	PLA	52	0.05
Formulation 2	PLGA	10	0.1
	PLA	6.2	0.45
	PLA	70	0.4
Formulation 3	PLA	9	0.55
	PLA	23	0.4

[a] The blend ratio of CDDP was 0.05 at all formulations.

2.4. Dissolution characteristics in vivo

Animal experiments were carried out in accordance with the ethical guidelines established by the Animal Experimental Ethical Committee of Tanabe Seiyaku Co., Ltd. Male Donryu rats (7 weeks of age, SLC, Shizuoka, Japan) were anesthetized by inhalation of diethyl ether. CDDP-MS was suspended in an aqueous vehicle (0.5% sodium carboxymethyl cellulose, 0.1% Tween 80 and 0.9% NaCl). The CDDP was dissolved in saline (CDDP-SOL). The CDDP-MS and CDDP-SOL were administered subcutaneously (s.c.) in the dorsal cervical region of the rats at a dose of 5.0 mg/kg. Blood was taken periodically from the jugular vein with a heparinized syringe. Furthermore, the CDDP-MS was recovered periodically from administered site. The platinum (Pt) concentrations in plasma and in recovered MS were determined by flameless atomic absorption spectroscopy. For determination of Pt concentrations, the samples were added to an acidic mixture (nitric acid:perchloric acid:-sulfuric acid = 24:24:1), and heated at 200°C for 2 h. Pt was then extracted with methyl isobutyl-ketone (Katayama Chemical, Osaka, Japan) after chelating with ammonium-pyroridine-carbamate (Wako Pure Chemical, Osaka, Japan). Pt concentrations were determined using a model Z8200 atomic absorption spectrometer (Hitachi 9000Z, Hitachi, Tokyo, Japan). A seven-stage heating program was used, consisting of drying at 50–140°C for 40 s, drying at 140°C for 10 s, ashing at 600–800°C for 5 s, ashing at 1000°C for 25 s, atomizing at 2700°C for 10 s, and conditioning at 2800°C for 5 s. The in vivo dissolution rate (%) was calculated by using the following equation:

$$\text{Dissolution}(\%) = (1 - \text{residual Pt amount/administered dose}) \times 100.$$

2.5. Systemic toxicity

The BDF1 mice (5 weeks of age, 21–25 g, Japan SLC, Shizuoka, Japan) were divided into 24 groups. Each group was composed of eight mice. Sixteen groups received CDDP-MS, six groups received CDDP-SOL, and two

control groups received either aqueous vehicle (0.5% sodium carboxymethyl cellulose, 0.1% Tween 80 and 0.9% NaCl) or drug-free MS. In the CDDP-MS groups, 10–80 mg/kg of CDDP was given i.p. at five or six dosages for each of the three formulations used. In the groups that received CDDP-SOL, 3.5–14 mg/kg was given i.p. at six dosages, increasing serially at a ratio of 1:1.14. The control group received drug-free MS (2.1 g/kg as MS), a dosage of MS greater than that which was contained in CDDP-MS at a CDDP dose of 80 mg/kg. All groups and the control groups received vehicle only were administered by the vehicle dose of 20 mL/kg. The mice were maintained under standard conditions and fed on standard mouse chow and tap water freely. The body weight of every mouse was observed for 120 days after drug administration as an indicator of systemic toxicity. Weight loss was calculated as a ratio to the initial body weight. Maximal tolerable dose (MTD) was defined as the dose resulting in a drop in body weight to 80% of initial weight. The MTD was calculated from maximum body weight loss versus dose profile using regression analysis by the least squares method.

2.6. Pharmacokinetic analysis

Pharmacokinetic parameters were determined from the plasma concentration versus time data. Any Pt concentrations of plasma below the lowest standard (20 ng/mL) were considered to be zero. The maximum plasma concentration (C_{max}) and the time to reach the maximum concentration (T_{max}) were read directly from the plasma concentration versus time data. The area under the curve of plasma concentration versus time from time zero to the time of last measurable plasma concentration point (AUC_{0-t}) was determined according to the trapezoidal rule. The bioavailability (F) was calculated by dividing the AUC_{0-t} of the CDDP-MS formulation by the AUC_{0-t} of CDDP-SOL.

2.7. Data analysis

All statistical analyses were accomplished using the Statistical Analysis System software (SAS Institute Inc., Cary, NC). Statistical comparisons were performed with Student's t test. A P value of less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Dissolution characteristics of CDDP-MS in vitro

The compositions of the CDDP-MS formulations are summarized in Table 1. The formulations 1 and 2 were prepared by using PLGA and PLA, and the formulation 3 was prepared with two PLAs of different molecular weights. The dissolution profiles of CDDP from MS in vitro are shown in Fig. 1. The amount of CDDP released in the first 3 h, the 'initial burst', was less than 3% for all formulations. Thereafter CDDP was released from formulations 1, 2 and 3 for 1, 2 and 5 weeks, respectively. It is considered that the

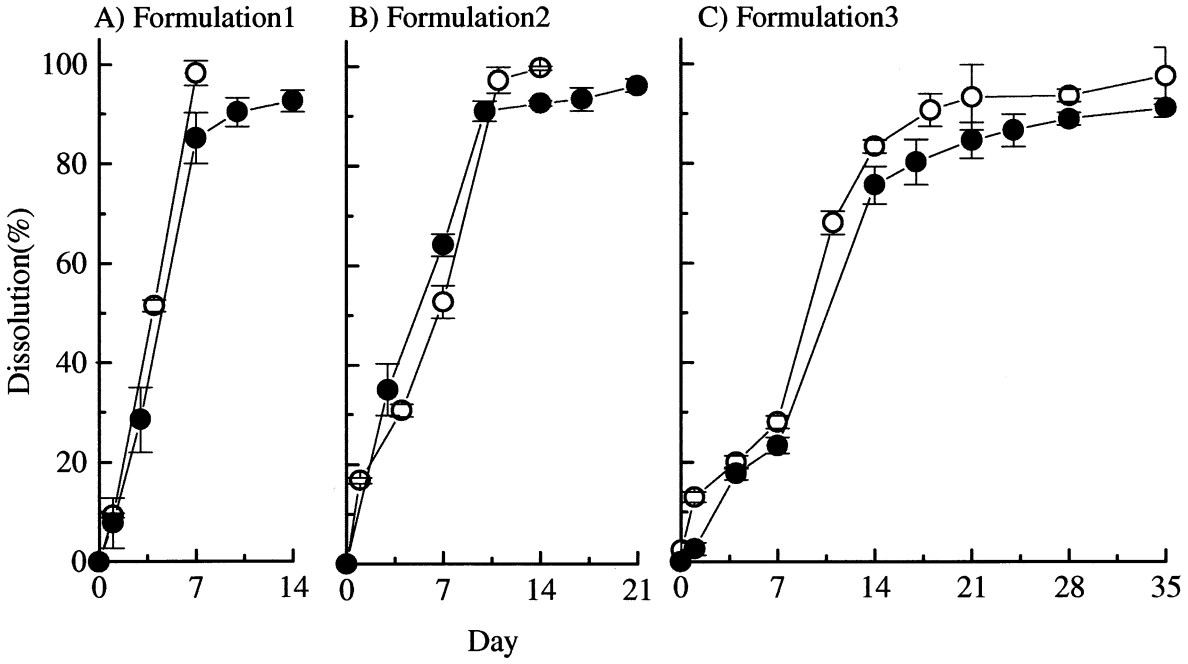

Fig. 1. Comparison of the dissolution profile of CDDP-MS in vivo (●) with in vitro (○). For in vitro dissolution testing, 10 mg of microspheres was weighted out in test tubes. The test tubes were filled with 10 mL of phosphate buffered saline (PBS, 0.15 M, pH 7.4) and then stirred at 25 rpm at 37 ± 1°C. The dissolution profiles in vivo were calculated from the residual CDDP amounts in microspheres at each time point. Each data point represents mean ± SD of four rats.

initial burst was due to rapid dissolution of the drug around the MS surface. Because the CDDP distributed in the internal of MS (multi-reservoir type MS), we prepared in this study, these MS have no initial burst [10]. The conventional CDDP-MS has been prepared by an oil-in-oil emulsion method, i.e. CDDP and polymer was dissolved in dimethylformamide and this oil phase was emulsified in liquid paraffin, resulting in non-crystal CDDP in MS [8,9]. However, in our study, CDDP-MS was prepared with a solid-in-oil-in-water emulsion method by using pulverized CDDP crystals, resulting in CDDP crystals imbedded in the MS. Consequently, it is suggested that the CDDP-MS would have no initial burst. Release of encapsulated molecules from MS occurs by two typical mechanisms: (1) release of the drug by diffusion through pores formed in the polymer matrix after hydration and (2) release of the drug as the polymer hydrolyzes and the MS degrades [11]. In general, higher the molecular weight of polymer is used, the slower is the formation of the pores in polymer matrix and slower the polymer degradation. Thus the longer release times observed by formulations 1, 2 and 3, in this order, can be explained using higher molecular weight shown in Table 1.

3.2. Comparison between CDDP dissolution in vivo and in vitro

Fig. 1 also shows the comparison of the dissolution profiles of CDDP-MS in vivo and in vitro. The formulations 1, 2 and 3 released CDDP during 1, 2 and 5 weeks in vivo as well as in vitro, respectively. Moreover the release rate in vivo was in accord with the release rate in vitro. This result indicates that the in vitro dissolution test of CDDP-MS was a reasonable estimate of CDDP release in vivo. In another study, Heya et al. evaluated the in vitro and in vivo release of thyrotrophin releasing hormone (TRH) from MS and found a correlation between the in vivo release rate following s.c. administration and the in vitro dissolution rate in 33 mM phosphate buffer (pH 7) [12]. In a present study, 9.57 mM phosphate buffered saline (pH 7.4) was used as the dissolution medium. Thus, the dissolution medium of CDDP and TRH was similar.

Whereas in case of the hydrophobic drug-containing MS composed of hydrophobic polymer, the release rate was predictable when water is used as the dissolution medium [13]. For MS containing hydrophobicity drugs, a close correlation between the degradation of the polymer and the drug release has been reported [14]. Basically the release proceeds accompanied by the degradation of the polymer regardless of the drug solubility incorporated in MS. However, with an increase in drug solubility, the release rate of the drug becomes much more rapid than the polymer degradation [15]. Because CDDP and TRH are relatively high water-soluble drugs, it is considered that release rate is more rapid than the polymer degradation, that is, the diffusion is more predominant factor for release rate rather than polymer degradation. Because the diffusion of hydrophilic drug in polymer is affected by pH, ion strength (osmotic pressure) and ion species [12], it might be necessary for CDDP to select the dissolution medium, which has similar ion strength and pH as biological fluid.

3.3. Plasma concentration of Pt

To determine the effect of different dissolution profiles in vivo and in vitro on the plasma concentration, the Pt concentration in plasma following s.c. administration of CDDP-MS was evaluated. Plasma Pt concentrations versus time profiles after s.c. administration of three formulations of CDDP-MS and CDDP-SOL are shown in Fig. 2. The Pt concentration after administration of the CDDP-SOL reached up to 5 μg/mL, thereafter, plasma Pt concentration decreased following a biphasic profile. On the other hand, Pt concentrations resulting from the CDDP-MS formulations were sustained without an initial high plasma concentration. The Pt concentrations of formulations 1 and 2 were gradually increased until 7 and 10 days, respectively, and then decreased at the same rate as CDDP-SOL. In the case of formulation 3, a Pt concentration of approximately 0.1 μg/mL was maintained for 17 days following administration and thereafter was found to decrease slower than CDDP-SOL. This rate of decrement suggested that dissolution of CDDP from MS continued for 35 days.

The pharmacokinetic parameters are summarized in Table 2. The C_{max} values of formulations 1, 2 and 3 were predictably decreased, and T_{max} values were increased in that order. These results suggest that the plasma Pt profiles reflect the dissolution profile in vivo as shown in Fig. 1. Thus, the plasma concentration of Pt is well controlled. The absorption rate to systemic circulation following s.c. administration was often reported to be slower than intraperitoneal and intrapleural administration [16,17]. However, the absorption rate of CDDP after s.c. administration was similar to that following intraperitoneal administration (data not shown). Consequently, it is suggested that

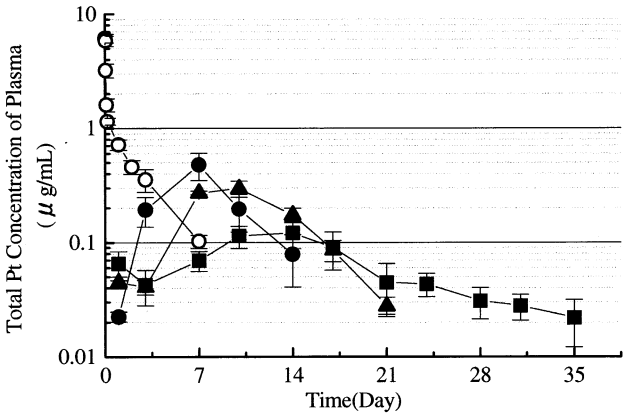

Fig. 2. Plasma Pt concentrations versus time profiles after subcutaneous administration of CDDP-MS, formulation 1 (●), formulation 2 (▲), formulation 3 (■) and CDDP-SOL (○) at a dose of 5 mg/kg to rats. Each data point represents mean ± SD of four rats.

Table 2
Pharmacokinetic parameters of microencapsulated CDDP[a]

Formulation	Dose (mg/kg)	T_{max}[b] (day)	C_{max}[c] (μg/mL)	AUC_{0-t}[d] (μg h/mL)	F[e] (%)	Residue (%)
Solution	5	–	–	77 ± 4	–	–
Formulation 1	5	7.0 ± 0.0	0.49 ± 0.11	72 ± 14	93 ± 18	7 ± 2
Formulation 2	5	9.3 ± 1.5	0.30 ± 0.03	64 ± 14	83 ± 19	4 ± 1
Formulation 3	5	13.0 ± 2.0	0.12 ± 0.03	64 ± 13	83 ± 17	9 ± 4
	8	14.0 ± 0.0	0.24 ± 0.04	103 ± 17	83 ± 14	12 ± 3
	10	14.0 ± 0.0	0.33 ± 0.05	133 ± 10	86 ± 7	12 ± 5
	20	15.5 ± 1.7	0.54 ± 0.13	280 ± 58	91 ± 19	12 ± 2

[a] Microsphere formulations are described in Table 1. All values are means ± SD. There were four animals in each group. All formulations were administered s.c. in the dorsal cervical region. Microspheres were suspended in vehicle and injected using 22-gauge needles.
[b] T_{max} values of formulation 1,2,3 at dose of 5 mg/kg are significantly different one another ($P < 0.05$). There are not significant differences along various doses of formulation 3.
[c] C_{max} values of formulation 1,2,3 at dose of 5 mg/kg are significantly different from one another ($P < 0.05$).
[d] AUC_{0-t} is the AUC from time 0 through the last sampling point (day 7 for solution group, day 14 for formulation 1, day 21 for formulation 2, day 35 for formulation 3).
[e] The bioavailability was determined by comparison of tie AUC_{0-t} values of formulations 1 ,2 and 3 to solution group.

the Pt levels after intraperitoneal administration of CDDP-MS was similar with that of s.c. administration.

Table 2 also shows the relationship between CDDP-MS dose and AUC_{0-t}. In this study, the CDDP-MS of formulation 3 was used. The AUC_{0-t} was proportionally increased with increasing dose and the correlation coefficient between doses and AUC_{0-t} was 0.9956. The C_{max} values were also proportional to dose, while the T_{max} values were little affected by dose. There was a possibility that the dissolution profile of CDDP at higher doses of CDDP-MS might change because the lower pH caused by degradation of PLA into lactic acid affect the dissolution rate of encapsulated drug. These results indicate that the CDDP dissolution from MS in vivo was little affected by the dose range from 5 to 20 mg/kg. The bioavailability (F) values, determined by comparison of the AUC_{0-t} values of three formulations to CDDP-SOL, were more than 83 and the sum of F and residual CDDP in MS were almost 100%. In addition, the AUC_{0-t} of CDDP-SOL after s.c. administration was the same as that following intravenous administration (data not shown). These results indicate that the majority of the CDDP released from MS was bioavailable to an extent similar to CDDP-SOL, and this result has potentially important implications for the anti-tumor efficacy of CDDP-MS. We consider that the CDDP-MS can be used for a regional chemotherapy. However, for systemic chemotherapy, CDDP-MS should have the same anti-tumor effect as CDDP-SOL with potentially lower C_{max} as shown in Fig. 2 and Table 1, because the anti-tumor effect of CDDP is dependent on AUC. Chemotherapy using CDDP has been carried out by various protocols [1,18,19], therefore formulations that have various dissolution period are necessary. We established three formulations of CDDP-MS, which provide a reliable Drug Delivery System to cancer chemotherapy.

3.4. Toxicity of CDDP-MS

Fig. 3 shows the effect of microencapsulation on the maximum body weight loss of mice injected with CDDP. The body weight of mice receiving vehicle and drug-free MS did not decrease during the experiment. The maximum body weight loss following CDDP-SOL was rapidly increased and reached 24% at 14 mg/kg. On the other hand, the body weight loss following CDDP-MS administration at dose of 14 mg/kg gave no change at all formulations. Moreover, the MTD, the dose resulting in a drop in body weight to 80% of initial weight, of CDDP-SOL was 13.4 mg/kg, whereas the CDDP-MS of formulations 1, 2 and 3 were 34.6, 44.2, 62.6 mg/kg, respectively. Thus, these three formulations of CDDP-MS resulted in decreased body weight loss in a dissolution rate dependent manner. These results suggest that the systemic toxicity of CDDP could be controlled by sustained release. Fig. 4 shows the

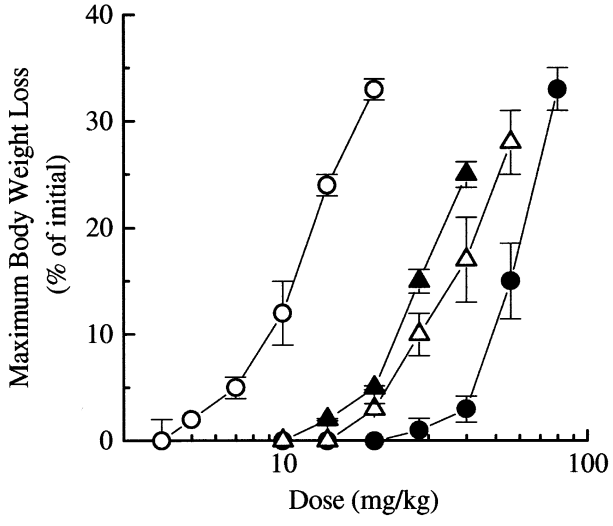

Fig. 3. Effect of microencapsulation on the maximum body weight loss of mice injected i.p. with CDDP. CDDP-SOL; ○, formulation 1; ▲, formulation 2; △, formulation 3 of CDDP-MS; ● Each data point represents mean ± SE of eight mice.

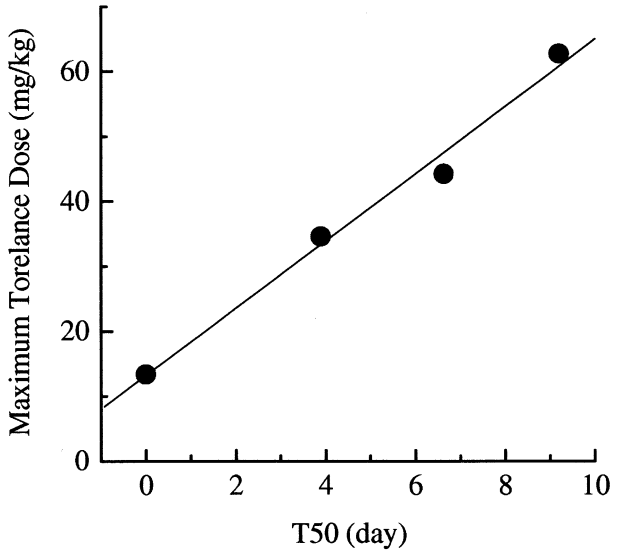

Fig. 4. Relationship between dissolution rate in vitro (T_{50}) and MTD of CDDP-MS. The T_{50} (dissolution rate) of CDDP-MS is the time at which 50% of CDDP release has occurred. The MTD, maximal tolerable dose, was the dose at which body weight loss was 20%. The equation and correlation coefficient calculated from the least squares method were $y = 5.22x + 13.2$ and $R^2 = 0.9935$, respectively.

relationship between the dissolution rate in vitro and the MTD of CDDP-MS. The MTD was proportionally increased with T_{50} and the correlation coefficient (R^2) was 0.9935. Thus, the systemic toxicity of CDDP-MS was predicted by evaluating the dissolution rate in vitro as the following equation:

$$\text{MTD (mg/kg)} = 5.22 \times T_{50(\text{day})} + 13.2$$

where T_{50} was the day when 50% of CDDP has been released from MS in vitro. The intercept value 13.2 of this equation indicates the MTD of CDDP-SOL. Furthermore, the slope of the equation was 5.22, which means sustained release of CDDP during 1-day leads to an increase in the MTD of 2.61 mg/kg. This correlation is attributed to the corresponding dissolution profiles in vitro and in vivo. This equation can only be used to predict the MTD of CDDP-MS when the CDDP-MS has little initial release. This is due to the fact that a large initial release is comparable to the administration of CDDP-SOL. It has been previously reported that the systemic toxicity of CDDP was reduced about two times by means of MS, which released CDDP over a period of 3 weeks after 40–50% dissolution until 1 day [20]. Our 1-, 2- and 5-week formulations of MS displayed about a two, three and five times reduction in the MTD compared to solution, respectively. Therefore, the systemic toxicity of CDDP-MS reported previously was the same as our 1-week type MS. Thus, a large amount of initial dissolution following administration might lead to serious systemic toxicity. Our CDDP-MS, however, had very low initial release (Fig. 1) and might therefore be associated with reduced systemic toxicity.

The dose-limiting factor of CDDP treatment is primarily renal toxicity. Therefore to clarify the toxicokinetics of CDDP, several experiments have been conducted. Campbell et al. have reported that patients with nephrotoxicity show significantly higher total platinum levels than non-nephrotoxic patients. Comparison of the pharmacokinetics of ultrafilterable platinum by rapid and prolonged dosing schedules, and the relationship between peak ultrafilterable platinum and the decline in creatinine clearance, suggest that maximum plasma concentration could be an important parameter for nephrotoxicity [21]. Furthermore, Nagai et al. have reported that the maximum blood urea nitrogen level as a renal failure indicator was related to an AUC of unchanged CDDP calculated from plasma concentrations greater than 0.9 μg/mL ($AUC_{0.9}$), a relationship described by the sigmoid maximum response model [22]. As shown in Fig. 2, the maximum plasma concentration of CDDP-MS was remarkably lower than CDDP-SOL and $AUC_{0.9}$ of unchanged CDDP was also markedly decreased by administration of CDDP-MS compared with CDDP-SOL in our pilot study (data not shown). These findings suggest that lower CDDP level in plasma after CDDP-MS administration results in lower level in kidney, inducing reduction in systemic toxicity.

4. Conclusion

Three formulations of CDDP-MS could be sustained for 1, 2 and 5 weeks, respectively, without a large amount of initial release in vivo that correlated well with the in vitro. The use of CDDP-MS resulted in a reduction in systemic toxicity compared with CDDP-SOL in a dissolution-rate dependent manner. The maximal tolerable dose of CDDP was proportionally increased with the day when 50% of CDDP had been released from MS in vitro ($\text{MTD} = 5.22 \times T_{50} + 13.2$, $R^2 = 0.9935$). We demonstrate that the systemic toxicity of CDDP-MS can be predicted by evaluation of the release rate in vitro.

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