Pharmacological and Pharmaceutical Properties of Freeze-Dried Formulations of Egg Albumin, Indomethacin, Olive Oil, or Fatty Acids

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Formulations consisting of egg albumin, indomethacin (IND), and olive oil or fatty acids, were prepared by vigorous stirring using a high-speed homogenizer and subsequent freeze-drying. To confirm the anti-inflammatory properties and ulcerogenic effects of the formulations, we examined the action of the formulations on carrageenan-induced edema in rats as well as their ulcerogenic actions in the same species. Compared with IND alone, albumin–IND–olive oil (9:1:4.3), albumin–IND–linolenic acid (9:1:4.3), albumin–IND–linolic acid (9:1:4.3), albumin–IND–oleic acid (9:1:4.3), albumin–IND–stearic acid (9:1:4.3), and albumin–IND–tristearin (9:1:4.3) formulations all exhibited a more potent inhibitory effect on carrageenan-induced edema. In addition, the inhibitory effects on edema formation of an albumin–IND (9:1) complex was as strong as that of IND alone. These results suggested that the bioavailability of IND was increased by olive oil, fatty acid, and tristearin as absorbefacient agents. The increase in the bioavailability was evident from the fact that the mean plasma levels, maximum plasma levels (C_{max}), and area under plasma concentration–time curve (AUC) values after oral administration of the albumin–IND–olive oil (9:1:4.3) formulation was significantly greater than that after administration of the drug alone. With respect to their ulcerogenic properties, the formulations were significantly less active than IND alone, suggesting that a reduction in the ulcerogenic activity of IND was by produced complexation with egg albumin.

Keywords egg albumin; olive oil; indomethacin; bioavailability; anti-inflammatory action; ulcerogenicity

Recently, natural and synthetic polymers have been evaluated for use as carriers able to enhance the bioavailability of drugs from oral and rectal dosage forms, or to reduce the incidence of adverse effects induced by various drugs during their administration.^{1–10} Seo *et al.* reported that the serum levels of spironolactone following oral administration of its cyclodextrin complexes were found to be greater than that after administration of drug alone,¹ and Nambu *et al.* and Espinar *et al.* also described the reduction in the ulcerogenicity of non-steroidal anti-inflammatory drugs (NSAID) by complexation with β -cyclodextrin.^{2,3} In addition, the use of some proteins as drug carriers has been studied as a mean of preparing solid dispersions.^{4–10} Macheras and Reppas were the first to prepare drug–milk freeze-dried formulations and demonstrated that these were promising as delivery systems for poorly soluble drugs because they were able to enhance their bioavailability.^{4,5} Imai *et al.* showed that the solid-dispersions of several poorly water-soluble drugs such as flurbiprofen, diclofenac acid, pindolol, diltiazem, and mefenamic acid with egg albumin and gelatin increased the dissolution rate and also enhanced the bioavailability of the drugs by the formation of water-soluble complexes.^{9,10}

Indomethacin (IND) is a poorly water-soluble NSAID which possess strong ulcerogenicity as a severe side-effect. In the present work, with the aim of both improving the bioavailability and reducing the ulcerogenicity of IND, we prepared freeze-dried egg albumin–IND formulations containing olive oil or fatty acids as an absorbefacient^{11–15} by means of a novel technique¹⁶ employing egg albumin for the solidification of liquids such as oils, fatty acids, and tocopherol, and then investigated their pharmacological and pharmaceutical properties.

Results and Discussion

The solid dispersion of poorly water-soluble drugs with

water-soluble proteins, such as egg albumin, gelatin, and milk casein, as carriers has been shown to enhance the solubility, dissolution rate, and bioavailability of these compounds by producing of water-soluble formulations.^{4–10} This suggests that, from a pharmaceutical point of view, such proteins may possess various advantages as drug additives. Therefore, we prepared a solid formulation consisting of egg albumin and IND (9:1), and examined its inhibitory effect on carrageenan-induced edema in rats compared with IND alone. As shown in Fig. 1, the inhibitory effects of the complex and IND alone were parallel and almost equal. The complex, at an oral dose of 3 mg/kg, as IND and the drug alone, at an oral dose of 3 mg/kg, both significantly inhibited the formation of paw edema at 2, 3 and 4 h after the injection of carrageenan. This suggests that

Fig. 1. Inhibitory Effects of an Albumin Complex and IND on the Edema (Rat Plantar) Induced by Carrageenan

●, control; ▲, IND; ○, albumin–IND (9:1) complex. Dosage was 3 mg/kg IND. Results are expressed as the $\bar{X} \pm \text{S.E.M.}$ of 5 rats. a) Significantly different from control, $p < 0.05$. b) Significantly different from control, $p < 0.01$.

in this particular case the bioavailability of IND may not have been improved sufficiently through complexation with egg albumin, to increase its pharmacological activity.

Recently, we developed a new solidification-technique for liquids such as oils and fatty acids using egg albumin.¹⁶⁾ We next exploited this technique to prepare a series of novel egg albumin-IND formulations containing olive oil or fatty acids which are known to have absorbefacient activity.¹¹⁻¹⁵⁾

Figure 2 shows the inhibitory effects of the albumin-IND-olive oil (9:1:4.3)¹⁷⁾ formulation and IND on carrageenan-induced edema in rats. The formulation, at oral doses of 0.3, 1, and 3 mg/kg as IND, reduced the

Fig. 2. Inhibitory Effects of Albumin Complexes and IND on the Edema (Rat Plantar) Induced by Carrageenan

●, control; ▲, IND 3 mg/kg; △, albumin-IND-olive oil (9:1:4.3) formulation 0.3 mg/kg as IND; □, albumin-IND-olive oil (9:1:4.3) formulation, 1 mg/kg as IND; ○, albumin-IND-olive oil (9:1:4.3) formulation, 3 mg/kg as IND. Results are expressed as the $\bar{X} \pm \text{S.E.M.}$ of 5 rats. a) Significantly different from control, $p < 0.05$. b) Significantly different from control, $p < 0.01$.

Fig. 3. Inhibitory Effects of Albumin Formulations and IND on the Edema (Rat Plantar) Induced by Carrageenan

●, control; ▲, IND; △, albumin-IND-linolenic acid (9:1:4.3) formulation; ○, albumin-IND-linoleic acid (9:1:4.3) formulation. Dosage was 3 mg/kg IND. Results are expressed as the $\bar{X} \pm \text{S.E.M.}$ of 5 rats. a) Significantly different from control, $p < 0.05$. b) Significantly different from control, $p < 0.01$.

formation of paw edema in a dose-dependent manner. Compared with IND alone, the inhibitory effect of the formulation, at an oral dose of 1 mg/kg as IND, was as potent as that of the drug alone, at an oral dose of 3 mg/kg, suggesting marked enhancement of the anti-inflammatory effect of IND by incorporating it in a preparation with egg albumin and olive oil.

The influences of egg albumin-IND formulations containing fatty acids on carrageenan-induced edema in rats were also evaluated and compared with the drug alone. As shown in Fig. 3, albumin-IND-linolenic acid (9:1:4.3) and albumin-IND-linoleic acid (9:1:4.3) formulations, at an oral dose of 3 mg/kg as IND, exhibited stronger inhibitory effects than IND alone. Moreover, the inhibitory effects of albumin-IND-oleic acid (9:1:4.3) and albumin-IND-

Fig. 4. Inhibitory Effects of Albumin Formulations and IND on the Edema (Rat Plantar) Induced by Carrageenan

●, control; ▲, IND; △, albumin-IND-oleic acid (9:1:4.3) formulation; ○, albumin-IND-stearic acid (9:1:4.3) formulation. Dosage was 3 mg/kg IND. Results are expressed as the $\bar{X} \pm \text{S.E.M.}$ of 5 rats. a) Significantly different from control, $p < 0.05$. b) Significantly different from control, $p < 0.01$.

Fig. 5. Inhibitory Effects of an Albumin Complex and IND on the Edema (Rat Plantar) Induced by Carrageenan

●, control; ▲, IND; ○, albumin-IND-tristearin (9:1:4.3) formulation. Dosage was 3 mg/kg IND. Results are expressed as the $\bar{X} \pm \text{S.E.M.}$ of 5 rats. a) Significantly different from control, $p < 0.05$.

stearic acid (9:1:4.3) formulations, at an oral dose of 3 mg/kg as IND, were more potent than that of the drug alone (Fig. 4). Figure 5 shows the inhibitory effects of albumin-IND-tristearin (9:1:4.3) complex and IND alone on the formation of edema induced by carrageenan. The inhibitory effect of this formulation was also stronger than that of the drug alone. These results clearly indicate enhancement of the anti-inflammatory effect by formulating the compound with olive oil or fatty acids as absorbefacient elements. In the above experiments, there was no significant difference in the anti-inflammatory effect between these formulations containing long chain triglycerides and fatty acids. From these experiments in rats, we would expect that these egg albumin-IND formulations containing olive oil or fatty acids would have good bioavailability following oral administration.

To confirm the improvement in the bioavailability of the drug, we next examined the plasma levels of IND following oral administration of the albumin-IND-olive oil (9:1:4.3) formulation, albumin-IND-stearic acid (9:1:4.3) formulation and IND alone. Figure 6 shows the IND concentration-time data following oral administration of the two formulations, at a dose of 10 mg/kg as IND and the drug alone at a dose of 10 mg/kg; the calculated pharmacokinetic parameters obtained from this data are summarized in Table 1. Significant differences in the mean plasma levels, the maximum plasma levels ($C_{\max}$), and the area under plasma concentration-time curve (AUC) values

TABLE 1. Summary of Plasma Profile Data Obtained after Oral Administration of IND and Its Albumin Formulations

Product administered	AUC ($\mu\text{g} \cdot \text{h}/\text{ml}$)	$C_{\max}$ ($\mu\text{g}/\text{ml}$)	MRT (h)	$T_{\max}$ (h)
IND alone	37.14 ± 5.06	3.99 ± 0.87	6.13 ± 0.73	1.7 ± 0.3
Albumin-IND-olive oil (9:1:4.3) formulation	68.29 ± 7.48^a	6.08 ± 0.68^a	5.99 ± 0.63	2.3 ± 0.7
Albumin-IND-stearic acid (9:1:4.3) formulation	75.82 ± 8.11^b	6.42 ± 0.65^a	6.99 ± 0.65	1.7 ± 0.6

Results are expressed as the $\bar{X} \pm \text{S.E.M.}$ of 5 rats. Dosage was 10 mg/kg IND in terms of IND. a) Significantly different from IND alone, $p < 0.05$. b) Significantly different from IND alone, $p < 0.01$.

Fig. 6. Plasma Concentrations of IND after Oral Administration of IND and Albumin Formulations to Rats

○, IND; ▲, albumin-IND-olive oil (9:1:4.3) formulation; ●, albumin-IND-stearic acid (9:1:4.3) formulation. Dosage was 10 mg/kg IND. Results are expressed as the $\bar{X} \pm \text{S.E.M.}$ of 5 rats. a) Significantly different from control, $p < 0.05$. b) Significantly different from control, $p < 0.01$.

were found comparing the drug alone and the two formulations. The AUC values of albumin-IND-olive oil and albumin-IND-stearic acid formulations up to 24 h post administration were 1.8 and 2 times¹⁸⁾ respectively greater than as that of the drug alone. On the other hand, there was no significant difference in the mean residence times (MRT), which may express the rate of bioavailability, and the $T_{\max}$, which may represent the time of absorption, between the two formulations and the drug alone. These findings obviously suggest that when administered orally to animals, the bioavailability of IND was greatly enhanced when in the form of protein-drug formulations containing absorbefacient elements such as olive oil or fatty acids, and this effect may be due to the increased absorption of the drug.

NSAID are well known to induce gastric mucosal lesions by producing a sharp reduction in the biosynthesis of endogenous prostaglandins which act as gastric resistant factors in gastric mucosa.¹⁹⁾ Recently, the complexation of NSAID with β -cyclodextrin has been reported to reduce these gastric mucosal lesions by preventing their direct stimulating action on gastric mucosa.^{2,3)} Therefore, our next step was to examine the ulcerogenicity of the egg albumin-IND complexes in rats.

The ulcerogenic actions of an albumin-IND (3:7) complex, an albumin-IND (9:1) complex, an albumin-IND-olive oil (9:1:4.3) formulation, and an albumin-IND-olive oil (9:1:4.3) physical mixture were assessed in comparison with the drug alone, and the results are shown in Fig. 7. The ulcer index in the group, IND alone, at an oral dose of 30 mg/kg was 18.8 ± 3.0 mm. The complexation with egg albumin markedly decreased the ulcerogenicity of IND in a manner proportional to the amount of egg albumin added. The ulcer indexes in the albumin-IND (3:7) and albumin-IND (9:1) complexes, at an oral dose of 30 mg/kg as IND, were 11.1 ± 2.8 and 7.2 ± 2.4 mm, respectively.

To investigate the influence of the addition of olive oil, the ulcerogenic effect of an albumin-IND-olive oil (9:1:4.3) formulation was examined. Compared with the albumin-IND (9:1) complex, the albumin-IND-olive oil (9:1:4.3) formulation exhibited approximately a 2-fold

Fig. 7. Gastric Lesions Induced by Albumin-IND Complexes, an Albumin-IND-Olive Oil Formulation, Its Physical Mixture, and IND in Rats

a, IND; b, albumin-IND (3:7) complex; c, albumin-IND (9:1) complex; d, albumin-IND-olive oil (9:1:4.3) formulation; e, physical mixture. Dosage was 30 mg/kg IND. Results are expressed as the $\bar{X} \pm \text{S.E.M.}$ of 5 rats. a) Significantly different from IND (a), $p < 0.05$. b) Significantly different from IND (a), $p < 0.01$.

decrease in ulcerogenicity, showing an ulcer index of 3.3 ± 1.2 mm. It is not readily apparent why olive oil promotes the reduction of IND-induced gastric ulceration by complexation with egg albumin. Further investigations on this action of olive oil are now in progress, directed at discovering the detailed mechanisms.

On the other hand, the ulcerogenic activity of the albumin-IND-olive oil (9:1:4.3) physical mixture and the drug alone as approximately equal. This clearly suggests that the ulcerogenicity of IND may be inhibited by the formation of a complex with egg albumin or egg albumin and olive oil.

We conclude that the freeze-dried egg albumin-IND formulations containing olive oil or fatty acids appear to offer promise as novel and beneficial pharmaceutical preparations of IND, demonstrating increased anti-inflammatory activity and reduced of gastric side effects when given orally.

Experimental

Materials Egg albumin, IND, olive oil, oleic acid, stearic acid, and tristearin were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Linoleic acid and linolenic acid were purchased from Nakalai Tesque, Inc. (Kyoto, Japan). Egg albumin (Wako practical grade, freeze-dried egg white albumin) was purchased from Wako Pure Chemical Industries, Ltd., and all other reagents and solvents were of analytical grade. λ -Carrageenan was supplied from the Zushi-kagaku Laboratory, Inc. (Kanagawa, Japan).

Animals Male Sprague-Dawley (SD) rats (200–250 g) purchased from Charles River, Japan, were used. They were housed in raised mesh-bottom cages under conditions of $22 \pm 2^\circ\text{C}$ temperature, $55 \pm 5\%$ humidity and 12 h light (from 7 a.m. to 7 p.m.), and given a commercial pellet diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum*.

Preparation of Formulations The test formulations were prepared according to the method described elsewhere¹⁶: IND, olive oil or fatty acids were dissolved in 25 ml of acetone, and then the mixture was added to 180 ml of 5% (w/v) aqueous egg albumin solution. The aqueous dispersion was vigorously stirred using a high speed homogenizer (BM-1, Nihonseiki Kaisha Ltd., Tokyo, Japan) at 2500 rpm for 2 h with cooling, evaporated under reduced pressure to remove the acetone, poured into a dish in the freeze-dryer (FD-80, Tokyo Rikakikai Co., Ltd., Tokyo, Japan), and freeze-dried. The solid freeze-dried complex was collected and passed through a stainless-steel sieve (100 mesh).

Preparation of the Physical Mixture The physical mixture was prepared by simple mixing of the albumin-olive oil (9:4.3) complex, prepared as described above, and IND in a ceramic mortar.

Determination of the IND Content in the Formulation Fifty mg of complex was sonicated in 20 ml of methanol to extract the IND completely. The recovery of IND from this extraction procedure was 98.2%. After filtration, the volume of solution was adjusted to 50 ml with methanol. The concentration of IND dissolved in the solution was determined by HPLC.

Chromatographic Conditions The chromatographic system consisted of a system controller (801-SC, Japan Spectroscopic, Tokyo, Japan), a HPLC pump (880-PU, Japan Spectroscopic), a sampler (850-AS, Japan Spectroscopic), a UV-detector (870-UV, Japan Spectroscopic), a data processor (C-R3A, Shimadzu, Kyoto, Japan). Separation was performed on a reverse-phase column (4.6 mm i.d. $\times$ 150 mm, Cosmosil 5C₁₈, Nacalai Tesque Inc., Kyoto, Japan). The analytical conditions were as follows: the internal standard solution was prepared by dissolving *n*-butyl *p*-hydroxybenzoate in methanol (62.5 ng/250 μ l). The mobile phase consisted of a mixture of acetonitrile and 0.05 M acetate buffer, pH 3.8 (1:1) at a flow rate of 1.0 ml/min and the detection wave-length was set at 254 nm. The mobile phase was prepared daily; it was filtered through a 0.5 μ m membrane filter before use.

Anti-inflammatory Activity Anti-inflammatory activity was assessed by inhibition of edema formation in the hind paw of rats in response to a subplantar injection of 0.1 ml of 1.0% λ -carrageenan. The experimental procedure followed that of Vinegar *et al.*²⁰ The test drug (100 mesh powder) was administered orally as a suspension in 1% carboxymethyl cellulose (sodium salt) solution 1 h before the injection of carrageenan.

The edema was measured by volume before and after the injection of carrageenan, and expressed as $(E_a - E_b)/E_b \times 100$, where E_b = volume of hind paw before the injection of carrageenan, and E_a = volume after injection.

Ulcerogenicity Before the experiments, rats were starved for 24 h, but allowed free access to water. Test drugs were given orally and 8 h later, the animals were killed by cervical dislocation. The stomachs were removed, inflated by injecting 10 ml of saline, and immersed in 1% formalin for 10 min. Each stomach was opened along the greater curvature, and the length (mm) of each necrotic lesion was measured by means of a dissecting microscope ($\times 10$) equipped with a scale. The sum of the length of all lesions was used as an ulcer index.

Bioavailability Studies Before the experiments, rats were fasted for 24 h, but allowed free access to water. Test drugs were administered orally, and then at different intervals (0.5, 1, 2, 4, 8, 24 h), 0.2 ml of blood was collected into heparinized tubes from the tail vein and centrifuged to obtain plasma samples which were frozen until assay. The concentration of IND in the plasma samples was determined according to a method described elsewhere²¹: to 50 μ l plasma, 200 μ l of distilled water and 25 μ l of 15% aqueous zinc sulfate were added, and then mixed by vortexing. After 5 min, 50 μ l of saturated aqueous barium hydroxide was added. To the mixture, 250 μ l of internal standard solution was added, mixed by vortexing, and centrifuged at 3000 rpm for 5 min. The supernatant (250 μ l) was then ready for direct HPLC analysis as described above.

Statistical Analysis Mean values of groups were expressed together with the standard error of the mean (S.E.M.). The significance of any differences in the data was evaluated using Student's *t*-test.

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