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

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To confirm the increased bioavailability of indomethacin (IND) when incorporated in a preparation with egg albumin and olive oil, we studied the detailed pharmaceutical characteristics of a ternary formulation consisting of egg albumin, IND and olive oil. From the results of X-ray powder diffraction measurements, the drug in the formulation was found to be in an amorphous form. When orally administered to rats, the ternary formulation significantly increased the plasma concentration and cumulative biliary and urinary excretion of IND alone as well as the urinary excretion of its major metabolite, desmethylindomethacin, compared with the drug alone. In addition, the dissolution rate of IND from the formulation was higher than that of the drug alone. These results clearly suggest that the bioavailability of IND was markedly improved by incorporating it in a protein-drug formulation containing olive oil as an absorbefacient element, and this effect may be due to an increased absorption of IND.

Keywords: egg albumin; formulation; indomethacin; olive oil; bioavailability; dissolution rate

It is well known that, when a poorly water-soluble drug is administered orally, the bioavailability is influenced by its dissolution rate. Therefore, many efforts have been made to increase the dissolution rate of poorly water-soluble drugs using natural and synthetic polymers as carriers.1-10 Recently, the use of some proteins as drug carriers has been reported for the preparation of solid-dispersions of several poorly water-soluble drugs.4-10 Macheras and Reppas were the first to prepare drug-milk freeze-dried formulations and demonstrated that these were promising as drug delivery systems for poorly water-soluble drugs aimed at enhancing their bioavailability.4,5 Imai et al. have reported that solid-dispersions of several poorly water-soluble drugs such as flurbiprofen, dichlofenac acid, pindolol, diltiazem and mafenamic acid with egg albumin and gelatin increased the dissolution rate and also enhanced the bioavailability of these drugs by the formation of water-soluble formations.9,10

Previously, we reported11 that both the inhibitory effects on the formation of paw edema induced by carrageenan and the plasma levels of indomethacin (IND) in rats following oral administration of freeze-dried egg albumin-IND formulations containing of olive oil or fatty acids were significantly superior to those observed when the drug was given alone; in contrast, the complex consisting of egg albumin and IND and the drug alone displayed approximately equal in anti-inflammatory activity. These findings clearly show that the bioavailability of IND was improved through the enhancement of its absorption by formulating it with olive oil or fatty acids as an absorbefacient.

In the present study, to confirm the improvement in the dissolution of IND by the preparation with egg albumin and olive oil, we examined pharmaceutical characteristics such as dissolution, biliary and urinary excretion and plasma drug concentrations after administration as the ternary formulation consisting of egg albumin, IND, and olive oil.

Results and Discussion

The X-ray diffractograms of the egg albumin–IND–olive oil (9:1:4.3) freeze-dried formulation, the egg albumin–IND–olive oil (9:1:4.3) physical mixture, albumin alone and the drug alone are shown in Fig. 1. The IND diffraction peaks were identified in the physical mixture and the drug alone (curves B and D, respectively). On the other hand, no peaks for crystalline IND were detected in the ternary formulation (curve C), indicating that the crystalline form had changed into the amorphous state when the formulated in the preparation with albumin and olive oil.

In the formulation, in which the molar ratio of albumin and IND is about 1:14, IND is presumed not to bind to specific site on albumin, but to disperse into the albumin–olive oil complex in amorphous state.

Figure 2 shows the concentration–time data for the drug following oral administrations of the formulation, at a dose of 10 mg/kg as IND, and the drug alone, at a dose of 10 mg/kg, and its pharmacokinetic parameters are also summarized in Table I. Significant differences were found in the mean plasma levels, the maximum plasma levels (Cmax) and the area under the plasma concentration–time curve (AUC) values between the formulation and the drug alone. The AUC value of the formulation up to 24 h post-administration was about twice that of the drug alone.

Fig. 1. Powder X-Ray Diffraction Patterns of Test Preparations

A, egg albumin alone; B, IND alone; C, egg albumin–IND–olive oil (9:1:4.3) formulation; D, egg albumin–IND–olive oil (9:1:4.3) physical mixture.
It is well known that the amorphous forms of drugs have a higher solubility and also a higher bioavailability compared with their crystalline forms. Furthermore, it has been reported that the intestinal absorption of poorly absorbable drugs is enhanced by glycerides, fatty acids and mixed micelles; olive oil is composed of various glycerides and its main component is the glyceride of oleic acid. From these findings, both the amorphous form of IND and the olive oil are thought to participate in the enhancement of the AUC following administration of the ternary formulation. On the other hand, there were no significant differences in the mean residence times (MRT), which describes the rate of bioavailability, and the T_{max}, which is selected to the time of absorption, between the formulation and the drug alone.

We next investigated the cumulative urinary excretion of IND and its major metabolite, desmethyldemethacin, following oral administration of the formulation and the drug alone in rats, and the results are shown in Figs. 3 and 4. Furthermore, the cumulative biliary excretion profile in rats was assessed, and these results are shown in Fig. 5.

The cumulative amounts of IND and desmethyldemethacin excreted in urine at 24, 48 and 72 h after oral administration of the test formulation were significantly greater than those after oral administration of IND alone. In agreement with the urinary excretion, the biliary excretion of the drug after oral administration of the test formulation was significantly greater than that after IND alone.

These results clearly indicate that when given orally to rats, the drug bioavailability was markedly improved by
incorporation into a protein-drug formulation containing olive oil, suggesting that this effect may be due to an increased absorption of IND.

To find out whether the improvement in the bioavailability of IND following formulation with egg albumin and olive oil resulted from a increase in the dissolution rate of the drug, we examined the dissolution of IND from the formulation in JP XI disintegration medium No. 1 (pH 1.2) and No. 2 (pH 6.8) at 37°C, and the results obtained are shown in Figs. 6 and 7. The ternary formulation exhibited a significantly greater dissolution rate than that of the drug alone in disintegration medium No. 2. On the other hand, the dissolution rates of both the formulation and the drug alone in disintegration medium No. 1 were very slow. The amount of IND passing through a cellulose membrane from the formulation in disintegration medium No. 2 was about twice as great as that from the drug alone, while both the formulation and the intact drug exhibited no dissolution in disintegration medium No. 1. Imaizumi et al. have reported that the dissolution rate of the amorphous form of IND was greater than that of the crystalline form in 5% methanol in water. The increased solubility may be the result of a decrease in crystallinity due to a change from the crystalline form of IND to the amorphous form in the formulation.

In conclusion, the data obtained in the present study clearly indicate that the bioavailability of IND was markedly improved by the formation of the freeze-dried protein-drug formulation, containing albumin as an efficient polymer support. The conversion of IND to its amorphous form and the action of olive oil as an absorbent component were responsible for the increased solubility and absorption of IND.

**Experimental**

**Materials** Egg albumin (Wako practical grade, freeze-dried egg white albumin), IND, olive oil and Freon 113 (1,1,2-trichloro-1,2,2-trifluoroethane) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Desmethyldiindomethacin and flurbiprofen were donated by Nippon Hypox Laboratory Inc. (Tokyo, Japan) and desmethyldinmethacin was recrystallized from aqueous acetone before use.

**Animals** Male Sprague-Dawley rats (200–250 g) purchased from Charles River, Japan, were used. They were housed in raised mesh-bottom cages under conditions of 22±2°C temperature, 55±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 Formulation** The test formulations were prepared according to the method described elsewhere. IND and olive oil 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 using a high speed homogenizer (BM-1, Nihonseiki Kaisha Ltd., Tokyo, Japan) at 25000 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 formulation was collected and passed through a stainless-steel sieve (100 mesh).

**Preparation of Physical Mixture** The physical mixture was prepared by simple mixing of an albumin–olive oil (9:4:3) formulation, prepared as described above, and IND in a ceramic mortar.

**Powder X-Ray Diffraction Studies** Powder X-ray diffraction analysis
was performed with a RAD-IIA diffraclometer (Rigaku Denki Company, Ltd., Tokyo, Japan). Operating conditions were as follows: X-ray source, CoKα radiation; voltage, 40 kV; current, 30 mA; receiving slit, 0.30 mm; scanning speed, 1°/min; angle, 2θ.

**Urinary Excretion of IND and Desmethylindomethacin** Urine samples from each rat were collected at 0 to 12, 12 to 24, 24 to 48 and 48 to 72 h after oral administration of the test drug given as a suspension in 1% carboxymethyl cellulose. Aliquots were assayed for IND and desmethyl-indomethacin and were counted, if appropriate.

**Biliary Excretion of IND** Bile cannulas were implanted in unanesthetized rats for bile collection. Bile was collected continuously for various time periods (2, 4, 6, 12, 24, 36, 48, 60 and 72 h) after oral administration of the test drug given as a suspension in 1% carboxymethyl cellulose.

**Analysis of the Concentration-Time Course in Rat Plasma after Oral Administration of the Formulation and IND** The rats were treated with a single oral administration of the albamine formulation and IND as a suspension in 1% carboxymethylcellulose. After administration, 0.2 ml of blood was collected at intervals from the tail artery using a heparinized syringe. The plasma was separated immediately by centrifugation at 3000 rpm for 10 min. To determine the drug concentration, 50 μl of plasma was submitted to HPLC.

**Sample Preparation** (a) Urine and Bile: To urine (1.0 ml) and bile (0.2 ml), 50 μl of internal standard solution, 0.7 ml of 0.3 N HCl-methanol and 1.4 ml of n-hexane-ethyl ether (1:1) were added and then vortexed. The sample solution was made biphasic by the addition of 0.7 ml of 0.1 N HCl, then centrifuged at 3000 rpm for 10 min. 1.0 ml of the organic phase were transferred to another tube and evaporated to dryness under a gentle stream of dry nitrogen at 40 °C. The residue was reconstituted in 0.2 ml of freon 113 and then 0.2 ml of buffer (pH 9.0) was added. The mixture was vortexed at 4 °C and centrifuged at 3000 rpm for 5 min. 0.1 ml aliquot of the aqueous phase was injected into the chromatograph.

(b) Plasma: To plasma (50 μl), 0.2 ml of distilled water and 25 μl of 15% (w/w) zinc sulfate solution were added, and then vortexed. After 5 min, 50 μl of saturated barium hydroxide was added. To the sample solution, 0.25 ml of internal standard solution was added, followed by vortexing and centrifugation at 3000 rpm for 5 min. The supernatant (0.25 ml) was then ready for direct HPLC analysis.

**Determination of IND Content in the Formulation** Fifty mg of the formulation was sonicated in 20 ml of methanol to elute IND completely. The recovery of the drug by means of this extraction procedure was 98.2%. After filtration, the volume 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 UV-detector (870-UV, Japan Spectroscopic), Integrator (CR-3A, Shimadzu, Kyoto, Japan). Separation was performed on a reverse-phase column (4.6 mm i.d. × 150 mm, Cosmosil 5C18, Nacalai Tesque Inc., Kyoto, Japan). The analytical conditions were as follows: (a) urine and bile; the internal standard solution was prepared by dissolving flurbiprofen in methanol (250 ng/50 μl). 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 1.5 ml/min and the detection wavelength was set at 280 nm. (b) plasma; the internal standard solution was prepared by dissolving n-butyral p-hydroxybenzoate in methanol (62.5 ng/250 μl). 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 wavelength was set at 254 nm. The mobile phase was prepared daily; it was filtered through a 0.5 μm membrane filter before use.

**Dissolution Study** The equivalent of 10 mg of drug, as a 100 mesh powder, was weighed and put in a beaker fitted with a jacket. The dissolution medium (20 ml) was maintained at 37 °C and stirred at 150 rpm. At appropriate intervals, 0.2 ml of solution was withdrawn through a cotton wool filter attached to a pipette, suitably diluted and assayed for drug by HPLC. The volume in the vessel was replaced with dissolution medium after each sampling.

**Permeation through a Cellulose Membrane** A dialysis cell was used in the cellulose membrane studies. The donor and acceptor compartment media (20 ml) were JP XI disintegration medium Nos. 1 and 2. The release of drug from the formation through the cellulose membrane was investigated in a dialysis cell at 37 °C. At appropriate times, 0.1 ml samples were withdrawn from the acceptor side for assay by HPLC and replaced with 0.1 ml buffer.

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

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**References**