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Gastrointestinal uptake of liposomes. II. In vivo studies

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Summary

The effect of liposomal entrapment on oral absorption of drugs was determined from the excretion profiles of entrapped markers after oral administration to rats. Liposomal entrapment does not facilitate the uptake of PEG-4000, a non-absorbable, water-soluble marker. The excretion patterns of hydrocortisone and salicylic acid, when entrapped in L-α-phosphatidylcholine distearoyl/cholesterol or egg lecithin/cholesterol multilamellar or unilamellar liposomes, were not significantly different from the excretion patterns of the unencapsulated drugs. These results are in agreement with in vitro and in situ studies which suggest that liposomal entrapment does not influence the intestinal absorption of drugs and, moreover, does not even protect entrapped drugs from the environment of the gastrointestinal tract. The combined in vitro, in situ and in vivo results strongly indicate that liposomes show little promise as a drug delivery system for oral administration. The increased oral bioavailability of liposomally incorporated drugs occasionally reported in the literature may be due to factors other than liposomal uptake.

Introduction

Attempts to use liposomes as an oral dosage form have been a major focus in drug delivery research in the past two decades. Since it has been suggested that liposomes may either protect the entrapped drugs from digestive degradation (Bangham et al., 1965) or increase the absorption of poorly absorbed drugs from the gastrointestinal tract (Sessa and Weissman, 1970), most of the studies in the literature are focussed on the effect of liposomal entrapment on oral absorption of drugs which are either poorly absorbed or drugs that are unstable in the gastrointestinal tract. For the most part, the results of these studies indicate that liposomal entrapment either does not facilitate oral absorption (Young et al., 1979; Kawada et al., 1981) or its influences on absorption are not predictable or reproducible (Arrieta-Molero et al., 1982; Tragl et al., 1979).

In a recent in vitro study on the stability of L-α-phosphatidylcholine distearoyl/cholesterol and egg lecithin/cholesterol multilamellar liposomes, we reported substantial loss of entrapped glucose and complete loss of entrapped carboxyfluorescein under conditions similar to what would be expected in the gastrointestinal tract. Also, transport studies using in vitro diffusion cells and everted sac experiments suggested that liposomal entrapment does not facilitate the transport of non-absorbable drugs and prevents the transport of absorbable ones. In situ studies seemed to confirm this viewpoint since almost all of the

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liposomes and their entrapped markers remain in the intestine even after 3 h (Chiang and Weiner, 1987).

On the other hand, there is a substantial body of literature which supports the views that liposomal entrapment offers protection for its encapsulated drug in the environment of the gastrointestinal tract (Rowland and Woodley, 1980) and facilitates the gastrointestinal transport of a variety of compounds (Nagata, et al., 1984; Dapergolas and Gregoriadis, 1976). Thus, despite two decades of research in this area, the utility of liposomes as an oral drug delivery system is still open to question.

The present work is an investigation of the effect of liposomal entrapment on in vivo absorption in the rat. In particular, we have sought insight into the following questions:

1. Do liposomes maintain their integrity in the physiological environment of the gastrointestinal tract?
2. Are non-absorbable markers (e.g., PEG-4000), when encapsulated into liposomes, able to traverse the gastrointestinal tract?
3. Does encapsulation into liposomes affect the gastrointestinal uptake of absorbable markers (e.g., salicylic acid and hydrocortisone)?

Materials and Methods

Materials

The lipids used were L-α-phosphatidylcholine distearoyl (Sigma Chemicals Co., St. Louis, MO), egg phosphatidylcholine (Avanti Polar Lipids, Birmingham, AL) and cholesterol (Sigma). L-α-Phosphatidylcholine distearoyl and egg lecithin were used as purchased and stored below 0°C. Cholesterol was recrystallized from ethanol solution 3 times. All liposome preparations that employed cholesterol were prepared using a phospholipid: cholesterol molar ratio of 2 : 1. The radiolabelled compounds that were used as entrapped markers, [^{14}C]PEG-4000, [^{14}C]salicylic acid and [^{3}H]hydrocortisone were purchased from New England Nuclear (Boston, MS). All radiolabelled markers were diluted with their unlabeled counterparts. All other chemicals were of reagent grade and used as obtained.

Preparation of liposomes

L-α-Phosphatidylcholine distearoyl/cholesterol and egg lecithin/cholesterol multilamellar vesicles were prepared by the method described by Chiang and Weiner (1987). Briefly, the lipid mixtures (32 μmol phospholipid and 16 μmol cholesterol) were dried from a chloroform solution at room temperature in a 20 ml glass scintillation vial (Kimble, Toledo, OH). Hydrocortisone which was to be entrapped was dissolved together with the phospholipids in the organic solvent while PEG-4000 and salicylate were dissolved in the aqueous buffer. A thin film was formed on the sides of the vial by hand agitation during the drying process and residual solvent was removed by storing the vial overnight under vacuum. The dried lipids were then hydrated with 1.0 ml of 67 mM phosphate buffer solution (pH 7.0) at a temperature above the transition temperature of the constituent phospholipid (61°C for liposomes composed of L-α-phosphatidylcholine distearoyl/cholesterol and room temperature for liposomes composed of egg lecithin/cholesterol). Incubation of the lipid film with buffer was carried out for 30 min with intermittent vortexing. The suspension was then sonicated in a bath-type sonicator (Branson Cleaning Equipment Co., Shelton, CN) for 30 s at the same temperature at which the films were hydrated, and the resultant dispersion was allowed to equilibrate for at least 4 h at room temperature. The dispersion was passed through a 2.0-μm polycarbonate filter (Nucleopore Corp., Pleasanton, CA) so as to remove large aggregates. The suspension was then centrifuged 3 times at 10,000 rpm for 5 min to remove untrapped marker from the liposome preparations. After each centrifugation step, the supernatant was removed and the liposomes pellet was resuspended in 2 ml of fresh buffer. After the final centrifugation step, the supernatant was assayed to ensure complete removal of free marker.

L-α-Phosphatidylcholine distearoyl/cholesterol large unilamellar vesicles were prepared by the reverse-phase evaporation technique described by Szoka and Papahadjopoulos (1978). Briefly, the lipid mixture (48 μmol phospholipid and 24 μmol cholesterol) was placed in a 50-ml round-bottom flask and the mixture was then dissolved in an organic solvent containing 6 ml each of chloro-

form and isopropyl ether. Hydrocortisone which was to be entrapped was dissolved together with the phospholipids in the organic solvent while PEG-4000 was dissolved in the aqueous buffer. Two ml of buffer were added, and the resulting two-phase system was purged with N_2 and sonicated briefly (2–5 min) until the mixture became a well-mixed opalescent dispersion. The temperature of sonication was maintained at 61° C (4° C above the transition temperature of distearoylphosphatidylcholine). Finally, the dispersion was placed in a rotary evaporator and the organic solvent was removed under reduced pressure at 61° C. As the solvent evaporated, the mixture first formed a viscous gel followed by a loss in viscosity to yield a fluid dispersion. At this point, excess buffer (1.5 ml) was added and the solvents were allowed to evaporate for an additional 10 min on the rotary evaporator to facilitate removal of trace amounts of organic solvent. The dispersion was then removed from the rotary evaporator and was maintained for 30 min in a 61° C water bath. The suspension was then centrifuged 3 times at 10,000 rpm for 5 min to remove untrapped marker. After each centrifugation step, the supernatant was removed and the liposomal pellet was resuspended in 4 ml of fresh buffer. After the final centrifugation step, the supernatant was assayed to ensure complete removal of free marker.

Determination of excretion profiles of free and liposomally entrapped PEG-4000 and hydrocortisone

Male Sprague-Dawley rats weighing 325–375 g were fasted overnight after being kept in metabolic cages (Nalge Co., Rochester, NY) for 4 days prior to initiation of the experiment. A no. 16 biomedical needle (≈ 7 cm long) was fitted to a 5-ml syringe and the test preparations were drawn up through the needle into the syringe. The needle was then inserted into the rat's mouth and through its esophagus and one to two ml of the following preparations were administered through the needle:

1. Aqueous solutions of PEG-4000 (≈ 1 mg) or hydrocortisone (≈ 0.1 mg);
2. PEG-4000 (≈ 1 mg) or hydrocortisone (≈ 0.1 mg) entrapped in:
   a. L-α-phosphatidylcholine distearoyl/ cholesterol multilamellar liposomes;
   b. egg lecithin/cholesterol multilamellar liposomes;
   c. L-α-phosphatidylcholine distearoyl/ cholesterol large unilamellar vesicles.

The fasting period was ended 4 h after administration of the test preparations. Water was given ad libitum throughout the experimental period and urine samples were collected at various time intervals for 48 h and assayed for radioactivity.

Determination of excretion profiles of free and liposomally entrapped salicylic acid

Femoral vein cannulation (to induce urine output) and bladder cannulation (to facilitate collection of urine) were used for this phase of the study since the rapid pharmacokinetics of salicylic acid necessitated frequent collection of urine samples. Male Sprague–Dawley rats, weight 330–370 g, were anesthetized by injection with sodium pentobarbital (50 mg/kg) i.p. Following the induction of anesthesia, the rats were fastened to an operating table which was covered with a temperature-controlled heating pad. 20% ethyl carbamate was administered as needed during the experimental period to maintain an anesthetized state.

The catheter used for femoral vein cannulation was prepared by fitting a 25-cm section of PE-50 tubing (Clay Adams, Parsippany, NJ) onto a 23-gauge needle attached to a 1-ml disposable syringe. The other end of the tubing was beveled to facilitate insertion into the femoral vein and the system was flushed with sodium heparin solution followed by normal saline. Following removal of the syringe the needle, connected to the polyethylene tubing, was attached to a three-way stopcock which was, in turn, placed directly on the hub of a 20-ml syringe containing the infusion solution.

A small groin incision was made with scissors and the femoral vein was exposed by rending the fat with the aid of forceps. The vein was then carefully separated from the remaining fascia using blunted forceps and two ligatures were placed under the femoral vein. The ligature closest to the distal end of the vein was pulled, tied tightly and taped firmly to the surgical table to keep the vessel taut. The second ligature was positioned at the proximal end of the vessel. After a small cut

was made in the vein between the two ligatures, and the catheter was introduced into this opening and eased into the vein, the second ligature was tied firmly around the vessel and the catheter. The area was then covered with gauze and kept moist with normal saline.

For the bladder cannulation, a midline incision was made in the abdomen of the rat to expose the bladder and urine was drained from the bladder using a 1-ml syringe with a 23-gauge needle attached. A purse-string suture was then made approximately 0.5 cm from the apex of the bladder using a 1/2 circle point needle (The Torrington Co., Torrington, CN). A small cut was made in the center of the suture area and a 10 cm section of PE-90 tubing, flanged at one end to prevent the cannula from slipping out of the bladder, was inserted. The suture was then pulled tightly and tied and the bladder was covered with gauze and kept moist with normal saline.

Following the surgical procedures described above, 0.9% saline containing 4% mannitol in a 20-ml syringe was infused via the femoral vein with a Harvard Apparatus infusion pump (Harvard Apparatus, Millis, MS). The infusion rate was 0.2 ml/min for the first 10 min and then adjusted to 0.1 ml/min throughout the remainder of the experiment. These rates were found to facilitate reasonable urine outputs (ca. 3–4 ml/h).

The following preparations were then administered in the manner described in the previous section:

1. Aqueous solution of salicylic acid (≈ 0.1 mg);
2. Salicylic acid (≈ 0.1 mg) entrapped in L-α-phosphatidylcholine distearoyl/cholesterol multilamellar liposomes.

Urine samples were then collected every 30 min for 4 h and were assayed by scintillation counting.

Results and Discussion

Upon oral administration of either free or liposomally entrapped PEG-4000, a water-soluble, non-absorbable marker, no radioactivity was detected in the urine, even after 48 h. The inability of liposomal encapsulation to facilitate uptake of this marker is in agreement with in vitro intestinal everted sac experiments (Whitmore and Wheeler, 1979; Seiden and Lichtenberg, 1979) and in situ studies using the Doluisio technique (Schwinke et al., 1984). On the other hand, in vitro experiments using everted sacs (Rowland and Woodley, 1981; Kimura et al., 1984) and isolated perfused intestinal loop systems (Patel et al., 1985), have led a number of investigators to suggest that liposomal entrapment of macromolecules may facilitate their intestinal absorption by an endocytosis mechanism. Chiang and Weiner (1987) point out that the evidence does not support endocytosis as a significant absorptive pathway, even if the liposomes retained their entrapped markers in the gastrointestinal tract.

Hydrocortisone and salicylic acid were chosen as markers because they represent drugs that are well absorbed and whose pharmacokinetics are defined in the rat (Wyngaarden et al., 1955; Yue and Varma, 1982). Furthermore, they are available as radiolabelled compounds and their intestinal uptake can be monitored by determining the cumulative percentage of radioactivity excreted in the urine as a function of time (Kirkpatrick et al., 1983; Yue and Varma, 1982).

The excretion profiles for free and liposomally entrapped hydrocortisone are shown in Fig. 1. Approximately 15% of the radioactivity (free hydrocortisone and metabolites) was recovered in the urine within two days after oral administration of free or liposomally entrapped hydrocortisone. Most of the radioactivity appeared within the first 24 h. The low percentage of recovery in urine is in agreement with data reported by Wyngaarden et al. (1955) who showed that after subcutaneous administration of radiolabelled drug to normal rats, most of the hydrocortisone and its metabolites appear in the feces as a result of transport to the gut via bile.

The liposomal type used (multilamellar or large unilamellar vesicles) and the constituent lipid composition (distearolphosphatidylcholine or egg lecithin) did not affect the excretion pattern. More importantly, liposomal encapsulation has no effect on the extent and rate of hydrocortisone absorption after oral administration as determined by one-way ANOVA ($P > 0.05$).

Since these results suggest that the various lipo-

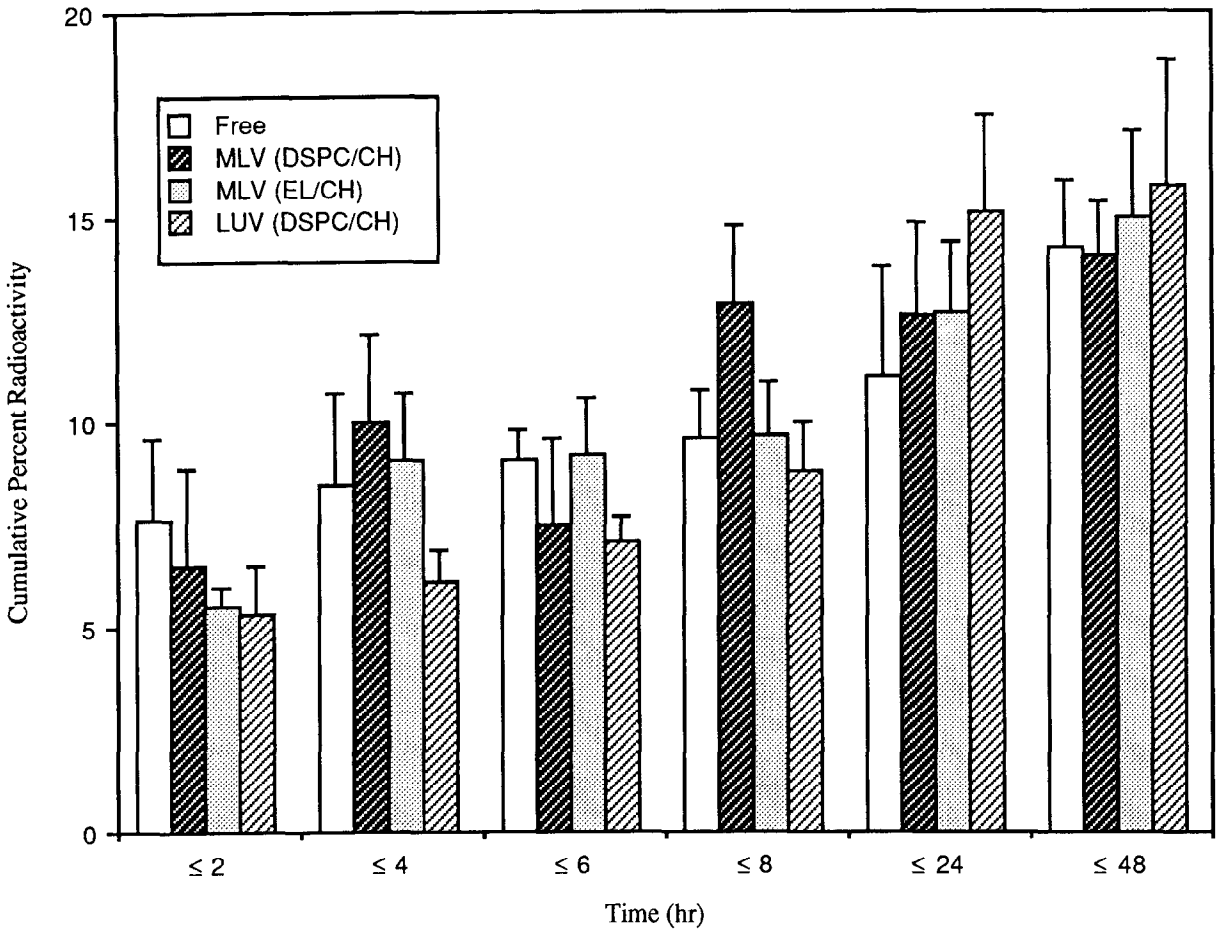

Fig. 1. Cumulative percentage of radioactivity excreted in urine after oral administration to rats of aqueous solutions of hydrocortisone or hydrocortisone entrapped in L-α-phosphatidylcholine distearoyl/cholesterol (DSPC/CH) multilamellar liposomes (MLV), egg lecithin/cholesterol (EL/CH) multilamellar liposomes or -α-phosphatidylcholine distearoyl/cholesterol large unilamellar vesicles (LUV). Bar denotes S.E.M. See text for experimental details.

somal preparations used were not stable in the gastrointestinal tract resulting in rapid leakage and subsequent absorption of free hydrocortisone, the salicylic acid studies were carried out by incorporating the marker into liposomes reported to be most stable in the environment of the gastrointestinal tract (Rowland and Woodley, 1980), i.e., L-α-phosphatidylcholine distearoyl/cholesterol multilamellar liposomes. The excretion profiles for free and liposomally entrapped salicylic acid using cannulated rats are shown in Fig. 2. Again, liposomal encapsulation has no effect on the extent and rate of salicylic acid absorption after oral administration as determined by the Student's *t*-test ($P > 0.05$).

Based on the similarity of the excretion profiles of unentrapped and entrapped hydrocortisone and salicyclic acid, it appears that the primary reason that liposomal encapsulation does not influence oral absorption is a result of the liposome's inability to retain entrapped marker in the gastrointestinal system. This view is supported by in vitro (Chiang and Weiner, 1987) and in vivo (Wu, et al., 1982; Mauk and Gamble, 1979; Hwang, 1983) studies which demonstrate the high degree of leakiness of diverse markers from liposomes in the

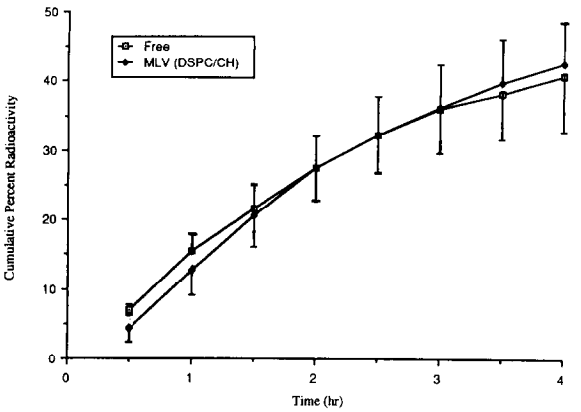

Fig. 2. Cumulative percentage of radioactivity excreted in urine after oral administration to cannulated rats of aqueous solutions of salicylate or salicylate entrapped in L-α-phosphatidylcholine distearoyl/cholesterol multilamellar liposomes. Bar denotes S.E.M. See text for experimental details.

gastrointestinal tract. Studies which report attempts to increase the gastrointestinal absorption of water-soluble drugs (Ueno et al., 1982), water-insoluble drugs (Nagata et al., 1984), ionophores (Young et al., 1979), and a variety of polypeptides and proteins (Hemker et al., 1980) further support the view that liposomal entrapment either does not facilitate oral absorption (Young et al., 1979) or affects absorption somewhat, but in a non-predictable manner (Jaskierowicz et al., 1985).

The large body of literature concerning the oral absorption of liposomally entrapped insulin is representative of the conflicting views on the ability of liposomal encapsulation to facilitate oral absorption. The emphasis on this drug can be attributed to the suggestion that liposomes can protect insulin from degradation in the gastrointestinal tract and thus reduce the blood glucose levels. For the most part, the data suggest that liposomally entrapped insulin does not lower blood glucose levels in normal subjects (Patel and Ryman, 1977; Kawade et al., 1981; Patel et al., 1982) and only about 1% of the administered dose of liposomally entrapped insulin appeared in the plasma within 60 min in normal human subjects (Patel et al., 1978). However, Dapergolas and Gregoriadis (1976) suggested that oral administration of liposomally entrapped insulin to normal rats produced a significant drop in blood glucose. Although hypoglycemia was observed after administering liposomally entrapped insulin to diabetic rats (Patel and Ryman, 1976; Hashimoto and Kawada, 1979), the reduction in blood glucose reported in the literature was neither dose-dependent nor easily reproduced (Arrieta-Molero et al., 1982; Tragl et al., 1979).

A number of related studies may help explain why a hypoglycemic effect is at times observed upon oral administration of liposomally entrapped insulin. Weingarten et al. (1985) found that insulin in solution was protected from enzymatic degradation upon incubation with empty liposomes, indicating that simple adsorption of insulin to the external phospholipid bilayer may protect it from proteolytic enzymes. Furthermore, Shichir et al. (1976) showed that various types of emulsions can assist the intestinal uptake of insulin. Thus, the hypoglycemia observed need not be dependent on the uptake of intact liposomes or even on insulin remaining entrapped in liposomes but may be the result of an interaction between insulin, lipids and bile salts.

There are other examples where liposomal entrapment of a drug appears to facilitate oral absorption. For example, Nagata et al. (1984), in their study of vitamin K absorption, reported that coagulation recovery following oral administration of liposomally entrapped vitamin K was faster than that for other preparations of the vitamin. The enhancement of vitamin K absorption can again be explained without resorting to a mechanism based on retention of drug in the liposome or gastrointestinal uptake of liposomes. Since the absorption of oil-soluble vitamins requires the presence of bile salts in the intestine, liposomal entrapment may simply increase access of the vitamin to the bile salt micelles and their subsequent micellar solubilization, thus facilitating the uptake of the vitamin.

The combined results of our in vitro and in vivo experiments demonstrate that liposomes do not influence the oral absorption of their entrapped drug. Furthermore, even the most "stable" liposomes do not retain entrapped marker in the gastrointestinal tract and hence will not even protect the drug from its hostile environment. The increased oral bioavailability of liposomally incor-

porated drugs occasionally reported in the literature may be due to factors other than liposomal uptake. Therefore, liposomes appear to have little promise as oral drug delivery systems.

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