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Coagulation recovery after warfarin-induced hypoprothrombinaemia by oral administration of liposomally-associated vitamin K_1 to rabbits

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The effect of liposomally-associated vitamin K_1, administered orally, was investigated using rabbits with warfarin-induced hypoprothrombinaemia, and evaluated in comparison with other dosage forms of the vitamin, including a vitamin K_1 emulsion, the physical mixture of the emulsion with empty liposomes, polyoxyethylene hydrogenated castor oil (HCO-60)-stabilized emulsion and the vitamin solubilized by HCO-60. The effect on blood coagulation recovery of each preparation was estimated as the time required for the prothrombin complex activity to return to 60% (TPCA60). The coagulation recovery time of the liposomal preparation was much faster than that of the other preparations and it was compared with the response to intravenous administration in which the vitamin was considered to be 100% available. The TPCA60 for the intravenous administration was 1·9 h, that for the oral liposomal preparation was 6·2 h, HCO-60 solubilized vitamin 13·6 h, HCO-60 stabilized emulsion 19·6 h, the physical mixture 17·8 h and plain emulsion 18·2 h. The vitamin K_1 dose was maintained at 12 mg kg^{-1} in each instance.

The oral administration of liposomally-associated drugs has attracted interest following reports that insulin entrapped in liposomes could cause a reduction of blood glucose levels in diabetic rats (Patel & Ryman 1976; Dapergolas & Gregoriadis 1977; Rustom et al 1979; Hemker et al 1980; Wu et al 1982). Whether liposome integrity is maintained and the vesicles can be absorbed in the digestive tract remains unclear (Rowland & Woodley 1981a, b; Deshmukh et al 1981).

Vitamin K is involved in the blood coagulation mechanism and is responsible for the synthesis of the vitamin K-dependent clotting factors II (prothrombin), VII, IX and X (O'Reilly 1980). Vitamin K deficiency causes a hypoprothrombinaemia distinctly marked by a prolonged prothrombin time and a tendency to bleeding.

According to the severity of bleeding, the vitamin is administered by oral, intramuscular and intravenous routes. There is no doubt that replacement of injection by oral ingestion would be of benefit to patients.

Warfarin is known to cause the accumulation of biologically inactive vitamin K_1 epoxide in the liver and to inhibit the vitamin K-dependent synthesis of coagulation factors (O'Reilly 1980). The process depends on at least two enzymes characterized as vitamin K_1 epoxidase and vitamin K_1 epoxide reductase and warfarin plays a role of the inhibitor of the vitamin K_1 epoxide cycle (Whitlon et al 1978). Park et al (1980) reported the effect of warfarin on PCA which suggests that a dose of 0·63 ng kg^{-1} (i.v.) was sufficient to block the synthesis of coagulation factors completely for at least 16 h in rabbits.

We have used warfarin-induced hypoprothrombinaemic rabbits as a means of assessing the absorption of efficiency of orally-administered vitamin K_1 by associating it with liposomes and we have compared the effect of this preparation with a plain vitamin K_1 emulsion, a hydrogenated castor oil (HCO-60)-stabilized emulsion and a solubilized form of the vitamin using HCO-60. The physical mixture of the plain emulsion and empty liposomes was also examined as a reference. The vitamin K_1 uptake efficiency was evaluated by the time required for the prothrombin complex activity (PCA) of the rabbits to return to 60% (TPCA60). All the orally-administered preparations were also compared with intravenous administration of the vitamin.

MATERIALS AND METHODS

Animals and materials

Male white rabbits, 3·0–4·0 kg, were maintained in a temperature-controlled room (25 °C) with free access to water and normal pelleted diet. Vitamin K_1 (Eisai Co. Ltd, Tokyo) was used as received. Warfarin (racemate) was prepared by dissolving its potassium salt (Eisai Co. Ltd, Tokyo) and used as an equimolar sodium salt. Vitamin K_1 injection

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(Hymeron-K_1, Toaeiyo Kagaku, Tokyo) and a modified thromboplastin reagent (Symplastin-A, Warner-Lambert, Morris Plains) were commercially obtained. Hydrogenated castor oil, trioxyethylated (HCO-60, Nikko Chemicals, Tokyo) was used as received. Phosphatidylcholine was extracted from egg yolk and its purity was confirmed by thin layer chromatography (Brandt & Lands 1967). All other chemicals used were of reagent grade.

Liposomally-associated vitamin K_1

The reversed-phase evaporation method was employed to disperse the vitamin K_1 which is a viscous oil (Szoka & Papahadjopoulos 1978). The chloroform stock solution containing 140 mg of phosphatidylcholine was taken into a 50 ml round-bottomed flask and the solvent removed by a rotary evaporator under reduced pressure. 9 ml of ether containing 50 mg of vitamin K_1 was then added to the flask, followed by 0·9% NaCl (saline) (3 ml). The resulting two phase system was briefly sonicated for 5 min in a bath-type sonicator until the mixture apparently became a uniformly dispersed emulsion. The emulsion was placed on a rotary evaporator and ether was removed at 36 °C. The resulting dispersion was diluted to a volume of 20 ml with saline. A typical liposomal stock preparation contains 180 μmol of phosphatidylcholine and 110 μmol of vitamin K_1 dispersed in 20 ml of the aqueous phase, in which liposomally-associated vitamin K_1 exists concurrently with the liposomally-free vitamin as droplets in the system. Because of practical difficulties in separating liposomes and oil droplets, their physical dimensions were examined by an optical microscope equipped with a Normarsky differential interference contrast attachment (Model BH-NIC, ×1000, Olympus Optical Co., Tokyo). The enlarged pictures (×3) showed that the morphology of the liposomes included spheres and doughnut-like shapes with a concave image and the size was widely distributed: the maximum was about 5 μm in diameter and the minimum was not determined. On the other hand, the average diameter of the concurrently existing droplets was about 2·8 μm.

The liposomal/free vitamin preparation was orally administered after being appropriately diluted with saline without separating the non-associated vitamin.

Vitamin K_1 emulsions and solution

Vitamin K_1 emulsions and solution were prepared as follows: (i) Plain emulsion: 50 mg of the vitamin was first dissolved in ethanol (1 ml) to which 20 ml of saline was added and the mixture vigorously shaken on a vortex mixer for 5 min. A similar optical microscopic observation showed that the size distribution ranged from 0·7 to 5 μm in diameter and the average size was tentatively 1·3 μm. Being physically unstable because no surfactant had been added, the emulsion was prepared immediately before being introduced into the stomach via a catheter. (ii) HCO-60 stabilized emulsion: HCO-60 (350 mg) melted at 40 °C was first dissolved in saline (18 ml) to which 1 ml of the vitamin ethanol solution (5%, w/v) was added and dispersed on a vortex mixer for 5 min. The final volume was adjusted to 20 ml. Unlike the plain emulsion, the primary droplets with relatively narrowly distributed size (1·5–2·8 μm) aggregated to form secondary particles consisting of 2–17 primary ones. (iii) HCO-60 solubilized vitamin: a vitamin ethanol solution (5%, w/v, 1 ml) was added to melted HCO-60 (350 mg). The resulting yellowish and transparent solution was diluted by adding saline so as to make a final volume 20 ml. (iv) Physical mixture of an emulsion and empty liposomes: this was prepared by mixing the plain emulsion and empty liposomes prepared in the same manner as above without the vitamin. This combination was selected because liposomes are likely to be destroyed to some extent if they are mixed with the surfactant-containing emulsion (Yotsuyanagi et al 1983). The lipid and vitamin ratio was maintained the same as the liposomal preparation of the vitamin.

Despite the same vitamin-surfactant ratio of the emulsified and solubilized systems (by HCO-60), both seemed unchanged on optical microscopic observation (×1000) for at least a week.

Gel filtration

A Sephadex G-50 column (1·9 cm i.d. × 45 cm, Pharmacia, Uppsala) was used to fractionate the liposomally-associated and free vitamin. Three ml of sample was applied on the top of the gel bed and eluted at room temperature (25 °C) with saline. The elution rate was 1·47 ml min^{-1} and the fraction of 2·5 ml each was collected. The void volume was determined by eluting blue dextran 2000 (Pharmacia, Uppsala). The HCO-60 solubilized preparation was eluted with HCO-60 solution in a similar way. Phosphatidylcholine was determined by the inorganic phosphorus assay (Eibl & Lands 1969). Vitamin K_1 was spectrophotometrically assayed at 249 nm after destruction of the vesicles by adding an equal volume of isopropyl alcohol to each eluted fraction.

Prothrombin complex activity (PCA)

The prothrombin time (PT) was determined by the method of Quick (1966). Blood samples (1·8 ml) were collected from the marginal ear vein into 3·8% trisodium citrate (0·2 ml) and centrifuged at 3000 rev min^{-1} for 10 min at room temperature (25 °C). 0·1 ml of citrated plasma was diluted with saline (0·1 ml) to half of which 0·2 ml of the thromboplastin reagent was added, and this was incubated at 37 °C for exactly 1 min. The clotting time was visually measured by trained eye. An average clotting time for five determinations was taken for each sample and the mean deviation was within 1·5% about the mean value in any measurements. To normalize PT, a standard curve of PCA for each rabbit maintained under normal conditions, i.e. warfarin-untreated condition, was constructed in a series of dilutions (0–100%) of plasma with saline (O'Reilly et al 1963). On the PCA standard curve, a PCA value of 60% as used by Yacobi et al (1974) was adopted as a criterion for coagulation recovery.

Warfarin-induced hypoprothrombinaemia

Before the experimental hypoprothrombinaemia was produced, the control PT of each animal was checked to confirm whether the PCA of the animal remained in the 60% clotting range compared with the standard curve of PCA. After that, animals were treated with warfarin (0·6–12·0 mg kg^{-1} i.v.). Blood samples (1·8 ml) were taken from the ear vein at appropriate intervals for determination of PT, which was converted to the corresponding PCA, until the PCA returned to normal. The time required for PCA to return to 60% was termed TPCA60. The relationship between PT and PCA and TPCA60 is shown in Fig. 1.

From the effect of warfarin doses on TPCA60, a dose of 12 mg kg^{-1} was selected for the induction of hypoprothrombinaemia in the rabbits, and this was characterized by a PCA value of 20% or less and usually took 22–24 h. The warfarin-induced condition was generally produced in each animal at intervals of at least three weeks to avoid possible enzyme induction or enzyme-related complication (Bjornsson et al 1979).

Experimental design of vitamin K_1 administration

Twelve rabbits were randomly used. A whole experimental procedure for each animal could be divided into two stages: (i) the induction of warfarin hypoprothrombinaemia; (ii) after the hypoprothrombinaemia had been established (i.e. after 24 h), various vitamin K_1 preparations were administered either intravenously or orally. The doses of the vitamin were as follows: vitamin K_1 injection (3·0–12 mg kg^{-1} i.v.), liposomal preparation (2·0–12 mg kg^{-1} oral) and other dispersed preparations (12·0 mg kg^{-1} oral). The volume of all of the oral preparations was adjusted to 20 ml with saline. Subsequently, the time course of the PT change, i.e. the PCA change, was followed until the PCA returned to normal. The restoration time (TPCA60) was regarded as a measure of the efficiency of the coagulation recovery.

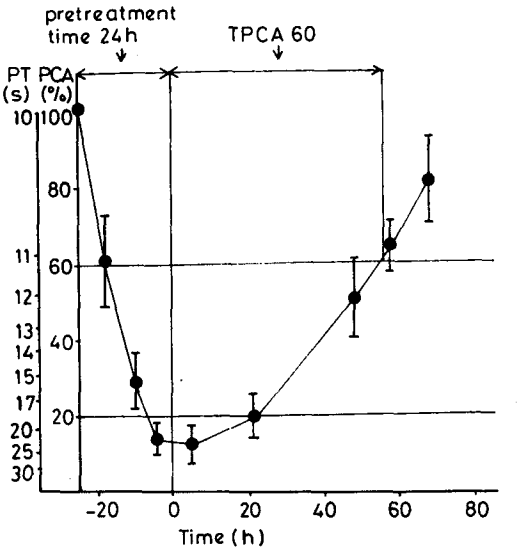

FIG. 1. The relationship between PCA (or PT) and TPCA60 and time course of PCA (or PT) after warfarin injection (12 mg kg^{-1} i.v.). Each point indicates the mean and s.e.m. (to the PCA scale) for 12 rabbits.

RESULTS

Warfarin-induced hypoprothrombinaemia

Using experimental hypoprothrombinaemia in rabbits, we investigated the effect of warfarin on PCA from which an appropriate dose of the anticoagulant and the pretreatment time required for the animals to reach a minimal level of PCA were determined.

Fig. 1 shows a typical example of the time course of the PCA change after the warfarin administration (12·0 mg kg^{-1} i.v.). The initial rate of decline of PCA was little affected by the dose of warfarin in the range of 0·6–12·0 mg kg^{-1}, but the magnitude of each trough of PCA and TPCA60 were dose-dependent (data other than 12·0 mg kg^{-1} not shown). The PCA values always remained more than 20% at doses less than 1·0 mg kg^{-1} and fluctuated above and below the PCA 20% level in the range of 1·0–3·0 mg kg^{-1}.

The relationship between the dose of warfarin and TPCA60 gave a sigmoid dose-dependent curve, approaching a maximal stage of TPCA60 asymptotically, as shown in Fig. 2. From these results, a dose of 12 mg kg^{-1} was chosen for the warfarin-treated test rabbits because of the relatively high reproducibility of the minimal PCA and the maximal inhibition of clotting factor synthesis.

After this dose of anticoagulant a time of 83·8 ± 12·4 h (n = 12) was required for a return to the PCA level 60%. As the TPCA60 was defined earlier, a pretreatment time of 24 h was allowed before initiation of the vitamin administration experiments; the TPCA60 for the spontaneous recovery was 59·8 ± 12·4 h.

Administration of vitamin K_1 preparations

The effect of an intravenous vitamin K_1 injection on the TPCA60 was examined to find how fast the PCA returned to 60%. In this instance the vitamin should be completely available compared with oral ingestion and therefore it should be possible to estimate the efficiencies, on coagulation recovery, of the various oral preparations by reference to the i.v. results. The dose-dependency of TPCA60 is illustrated in Fig. 3, in which the minimal TPCA60, i.e. the time required for the fastest recovery, was about 2 h. This was brought about by i.v. doses of more than 6·0 mg kg^{-1} of the vitamin.

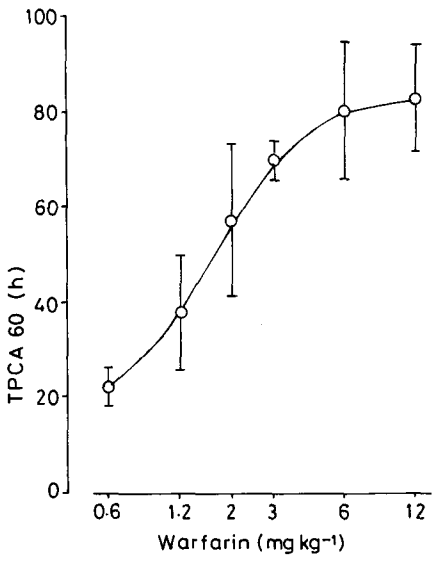

FIG. 2. Dose-dependency of TPCA60 in rabbits treated by warfarin. Each point shows the mean and s.e.m. for 5–12 rabbits.

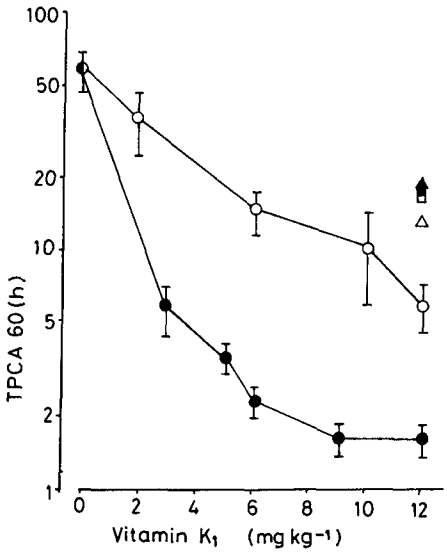

FIG. 3. Effects of various vitamin K_1 administrations on TPCA60. ◐, spontaneous recovery; ●, i.v. injection; ○, liposomal preparation; ■, plain emulsion; □, mixture of plain emulsion and empty liposomes; ▲, HCO-60 stabilized emulsion and △, HCO-60 solubilized solution. Each point indicates the mean and s.e.m. for 4–6 rabbits.

The dependency of the TPCA60 on the dose of the liposomal preparation is also shown in Fig. 3. Although the extent of recovery after the oral preparation was much less than after the injection, it can be seen that the liposomal preparation at 12·0 mg kg^{-1} was almost equivalent to the i.v. injection at 3·0 mg kg^{-1} on the TPCA60 scale, when the TPCA 60 was about 6 h.

The efficiency of the liposomal preparation in effecting recovery was then compared with those of the other oral preparations at the fixed dose of the vitamin of 12 mg kg^{-1} (Fig. 3). It is apparent that the coagulation recovery following the liposomal preparation was faster than that of the other oral preparations, and nearer that of the i.v. administration.

Since the vitamin in the liposomal preparation that was not incorporated in the liposomes, which mainly existed in a state of droplets, was not separated from the liposomal fraction at dosing, the problem arises as to how much vitamin is associated with liposomes. Unfortunately, it was not possible to quantitate the associated and non-associated vitamin separately because there was no clear-cut separation between the oil phase and the liposomal fraction even after ultracentrifugation (100 000*g*) probably because of the close densities of both phases (Mason & Huang 1978; JP X 1981), coprecipitation, and because the

sizes of the vesicles and oil droplets were too large for separation by gel chromatography, even when the original liposomal suspension was further diluted.

Instead, an attempt was made to estimate or approximate the amount of vitamin K_1 associated with liposomes as follows: the vitamin-phosphatidylcholine ratio of the original preparation was gradually reduced until extra droplets were not recognized by optical microscopy (×1000). No or few droplets were observed at the ratio of about 0·06. Although it is very probable that very fine droplets still exist in the system, the liposomal suspension could be loaded on a gel bed after processing by sonication, the apparently droplet-free liposomal preparation being ultracentrifuged at 10 000*g*, and the supernatant from this being sonicated for 1 min (probe type) and then applied to a Sephadex G-50 gel column.

Fig. 4 shows the chromatograms of vitamin K_1 and phosphatidylcholine. The vitamin was eluted with fractions 16–22 and 41–49 with remarkable resolution, the first peak (corresponding to the void volume) indicates the liposomally-associated vitamin and the second peak is assumed to be either solubilized vitamin or the reversibly adsorbed fraction (i.e. vitamin not incorporated in liposomes) collected on the gel bed. The complete separation does not always mean that the mechanism of retention of the vitamin is a size-exclusion phenomenon. Since no phosphatidylcholine was detected in the second peak of the vitamin, the peak is probably due to unincorporated vitamin.

The molar ratio (vitamin/phosphatidylcholine) of the first peak was found to be 0·04. If the magnitude of the vitamin association obtained is applicable to the original liposomal preparation, only 7·2 μmol of the vitamin would be accommodated in the liposomes (180 μmol as phosphatidylcholine). This value corresponds to only 6·5% of the vitamin added being incorporated in the present liposomal preparation.

The HCO-60 solubilized vitamin was also examined to find whether the vitamin was eluted in the corresponding fractions to the second peak. The chromatogram gave only one peak of the vitamin which appeared in the void volume (Fig. 5). This means that the vitamin is incorporated in a particulate system whose size is large enough to be excluded by Sephadex G-50 (fractionated range of mol wt 1500–30 000), therefore the vitamin is likely to be solubilized by HCO-60 micelles. Although the aggregation number of the surfactant is unknown, the size of the micelles could be considered to be relatively large compared with that of other nonionic surfactants (Fendler & Fendler 1975). Furthermore, the absence of the second peak is considered to be due to the extremely high partition of the vitamin to the micellar phase.

DISCUSSION

Table 1 indicates the efficiencies of various dosage forms represented by TPCA60. The liposomal preparation was clearly more effective than the other preparations ($P < 0·01$). The liposomal preparation

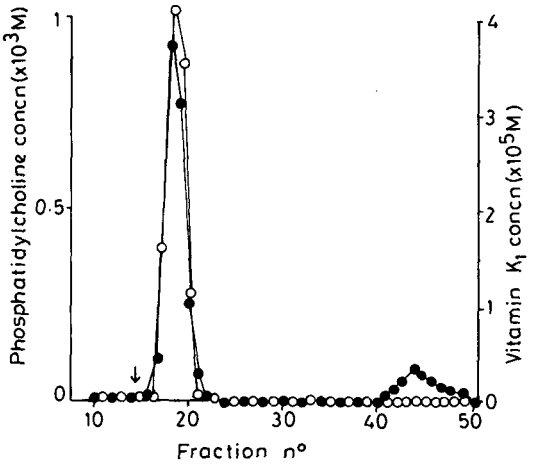

FIG. 4. Chromatography on Sephadex G-50 of the liposomally associated vitamin K_1. ↓ indicates the void volume. Eluent: saline; fraction volume: 2·5 ml. Recovery of phosphatidylcholine (○) and vitamin K_1 (●) were 94·9 and 102% respectively.

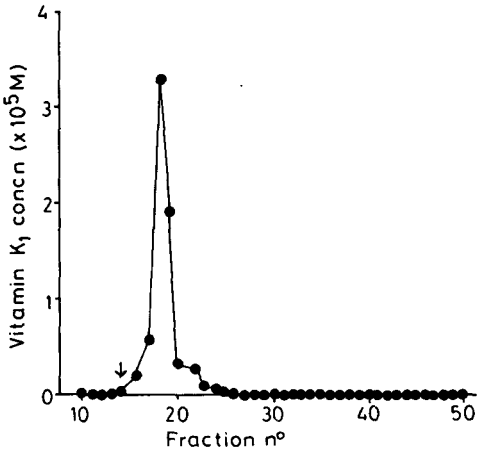

FIG. 5. Chromatography on Sephadex G-50 of the HCO-60 solubilized vitamin K_1. ↓ indicates the void volume. Eluent: HCO-60 solution ($1·9 \times 10^{-4}$ M); fraction volume: 2·5 ml. Recovery of vitamin K_1 was 101%.

shortened the TPCA60 by about 13 h compared with the emulsified systems and by about 7 h for the solubilized system. The improved recovery efficiency of PCA induced by the liposomal vitamin K_1 might be useful clinically for a special bleeding condition.

Table 1. Blood coagulation recovery of various vitamin K_1 preparations for warfarin-induced hypoprothrombinaemic rabbits.

Dosage form[a] of vitamin K_1	TPCA60 (mean ± s.e.m., h)	Paired *t*-test[c] *P*
None	59·8 ± 12·4 (n = 12)[b]	—
Intravenous: Vitamin K_1	1·9 ± 0·3 (n = 5)	—
Oral:		
Liposomal	6·2 ± 1·4 (n = 6)	—
Plain emulsion	18·2 ± 1·7 (n = 6)	<0·01
Physical mixture of plain emulsion and empty liposome	17·8 ± 1·7 (n = 5)	<0·01
HCO-60 stabilized emulsion	19·6 ± 3·3 (n = 4)	<0·01
HCO-60 solubilized solution	13·6 ± 2·0 (n = 4)	<0·01

[a] Each dosage form was administered at a 12 mg kg^{-1} basis.
[b] TPCA60 was calculated by subtracting 24 h from 83·8 ± 12·4 h.
[c] Test of significance: liposomal, to other oral administrations.

Little difference in TPCA60 was found among the emulsified systems including the mixture of the plain emulsion and empty liposomes. The HCO-60 stabilized emulsion and the plain emulsion seem to have entirely different size distributions in the gut since the latter is likely to coalesce easily after ingestion, and yet both had an almost identical TPCA60. This indicates that the size of the vitamin droplets may not be so important a factor, presumably because of the poor availability of the vitamin from the droplets in addition to the relatively large dose. The HCO-60 solubilized vitamin seemed slightly more effective than the emulsified preparations, $P < 0.05$ by the paired *t*-test.

Whilst the extent of association of vitamin K_1 with liposomes was found to be unexpectedly low, there was a distinct difference in the coagulation recovery between the liposomal preparation and the other dispersed systems and these results raise a question of the mechanistic role of liposomes for vitamin delivery.

It has been generally assumed that only molecularly dispersed species of substances could be transported across the intestinal mucosa and absorbed into the systemic circulation. However, Volkheimer (1973, 1975) suggested that absorption of particulate matter in the μm range occurred in the intestine especially at the tips of the villi and in the neighbourhood of goblet cells. Furthermore, the particles must be relatively hard since soft materials such as liquid droplets are thought not to penetrate intact through the intestinal mucosa (LeFevre & Joel 1977).

However, another mechanism might account for the observed 'enhanced' delivery in the intestine without postulating that liposomes are absorbed intact; i.e. there could be more effective adhesion to the surface of villi and easier formation of mixed micelles with cholic acids (Patel & Ryman 1981).

Whilst the present study does not yield information on mechanism of action of the liposomal preparation, being confined to show only the results of the faster coagulation recovery produced by the liposomal preparation of vitamin K_1, liposomal preparations appear to be promising carriers in the intestine even for relatively stable substances.

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