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Effectiveness and Toxicity Screening of Various Absorption Enhancers in the Rat Small Intestine: Effects of Absorption Enhancers on the Intestinal Absorption of Phenol Red and the Release of Protein and Phospholipids from the Intestinal Membrane

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Abstract

Sodium glycocholate, sodium taurocholate, sodium deoxycholate, EDTA, sodium salicylate, sodium caprate, diethyl maleate, *N*-lauryl-β-D-maltopyranoside, linoleic acid polyoxyethylated (60 mol) mixed micelles (all 20 mM) have been ranked in order of their effectiveness as enhancers of the absorption of drugs in the rat small intestine, by use of an in-situ loop model with phenol red as a model drug. Local toxicity in rats was examined by assessing protein and phospholipid release as biological markers.

Of the absorption enhancers, sodium deoxycholate, EDTA and *N*-lauryl-β-D-maltopyranoside were the most effective; sodium deoxycholate and EDTA, however, caused significant release of protein and phospholipids. *N*-lauryl-β-D-maltopyranoside, on the other hand, did not damage the small intestinal membrane. Sodium taurocholate enhanced phenol red absorption from the small intestine and resulted in little or no protein and phospholipids release. Sodium salicylate, diethyl maleate and the mixed micelles had no absorption-promoting effects on phenol red. There was good correlation between the area under the plasma concentration-time curve for phenol red and the amounts of protein and phospholipid released in the presence of absorption enhancers.

From these results it might be concluded that *N*-lauryl-β-D-maltopyranoside and sodium taurocholate are effective absorption enhancers which have low toxicity levels at a concentration of 20 mM.

A large number of absorption enhancers, including surfactants, bile salts, chelating agents and fatty acids, have recently been used to enhance the intestinal absorption of antibiotics and macromolecules (Muranishi 1990; Lee et al 1991). Murakami et al (1984) reported that the rectal absorption of sodium ampicillin was promoted by addition of various bile salts. Nishihata et al (1981) demonstrated that intestinal absorption of insulin was enhanced in the presence of 5-methoxysalicylate. We also found that monoolein-bile salt mixed micelles enhanced intestinal absorption of heparin, streptomycin, gentamicin and interferon-β, but that some of these adjuvants cause damage and irritate the intestinal mucosal membrane (Muranishi 1985). Although many studies have been performed to investigate toxicity by means of haemolysis, protein release or morphological observation (Murakami et al 1984; Tomita et al 1988; Anderberg et al 1993), few studies have been performed to investigate their effectiveness and toxicity in a single study.

In this study phenol red, a poorly orally absorbed drug which is stable in the gastrointestinal tract, was chosen as a model polar drug and we compared a variety of absorption enhancers in a single experimental system in order to rank their absorption-promoting ability. The absorption enhancers used were sodium glycocholate, sodium taurocholate, sodium deoxycholate, EDTA, sodium salicylate, sodium caprate, diethyl maleate, *N*-laury-β-D-maltopyranoside and linoleic acid-hydrogenated castor oil 60 (HCO60) mixed micelle. The concentration used, 20 mM, was selected to enable comparison of their promoting effects under similar conditions. We also evaluated local intestinal damage to select the effective absorption enhancers with low toxicity.

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Materials and Methods

Materials

Sodium glycocholate and sodium taurocholate were purchased from Sigma (St Louis, MO, USA). Phenol red and sodium deoxycholate were obtained from Wako Pure Chemical Industries (Osaka, Japan). Linoleic acid of high purity grade (> 99%) was kindly supplied by Nippon Oil & Fats (Tokyo, Japan). Diethyl maleate was obtained from Kanto Chemical Co. (Tokyo, Japan). Sodium salicylate and EDTA were obtained from Nacalai Tesque (Kyoto, Japan). HCO60 was a gift from Nikko Chemical Co. (Tokyo, Japan). *N*-lauryl-β-D-maltopyranoside was kindly supplied by Japan Fine Chemical Co. (Osaka, Japan). Sodium caprate was purchased from Tokyo Kasei Industries Co. (Tokyo, Japan). All other chemicals were of reagent grade.

Animal experiments

Absorption experiments were performed on male Wistar albino rats, 200–300 g (Japan SLC Inc., Hamamatsu, Japan) by an in-situ closed-loop method (Hashida et al 1984). Animals were fasted for 18 h before the experiments, with water freely available; during the experiments they were anaesthetized with sodium pentobarbital (30 mg kg^{-1}, i.p.). The ileocaecum junction was bound with a ligature and the drug solutions (2·5 mg mL^{-1} of phenol red in phosphate-buffered saline)

were injected into the small intestinal loop, which was immediately closed with a ligature below the part pierced with a needle (Terumo; 27 gauge × ¾"; 0·40 × 19 mm). Phenol red solution (2 mL) with or without absorption enhancers (20 mM) was introduced into the intestinal loop, which was closed by clipping with forceps at the cannulated positions of each tube. Rats were left for 1 h before administration of the drug on a hot-plate kept at 37°C. Blood samples were obtained from the jugular vein at predetermined times up to 240 min and the plasma concentration of phenol red was determined by spectrophotometric determination at 560 nm (Hitachi, Tokyo, Japan, model U-2000 spectrophotometer) after making the sample (200 μL) alkaline with 1 M NaOH (3 mL). The amounts of phenol red in the final solutions were used to calculate absorption percentages. The amount of drug that disappeared from the lumen was calculated as the difference between the amounts of phenol red in the initial and the final solutions.

Release of protein and phospholipids from the intestinal mucosa by absorption enhancers

The release of tissue protein and phospholipids from the intestinal mucosa was used as an index to assess tissue damage caused by the various absorption enhancers. Buffer solution (2 mL) with or without absorption enhancer (20 mM) was injected into the small intestinal loop in a manner similar to that used for the absorption experiment. Rats were left for 4 h after administration of the solution and at the end of this experiment the perfusate in the small intestine was withdrawn for assessment of the release of protein and phospholipids. The amount of protein released from the intestinal membrane was determined by the method of Lowry et al (1951) with bovine serum albumin as the standard. For sodium taurocholate, sodium salicylate and EDTA, the amount of protein released was determined by a modification of method of Bensadoun & Weinstein (1976) to avoid interference with the assay. The amount of phospholipids released from the intestinal membrane was determined with a Nescauto PL phospholipid assay kit (Nippon Shoji, Japan). Absorption enhancers used in this experiment did not interfere with the phospholipid assay.

Statistical analysis

Results were expressed as the mean ± s.e.m.; statistical significance was assessed using Student's *t*-test.

Results

Effects of various absorption enhancers on absorption of phenol red from the small intestine

Fig. 1 shows the time-course of the concentration of phenol red in plasma after its administration to the small intestine in the

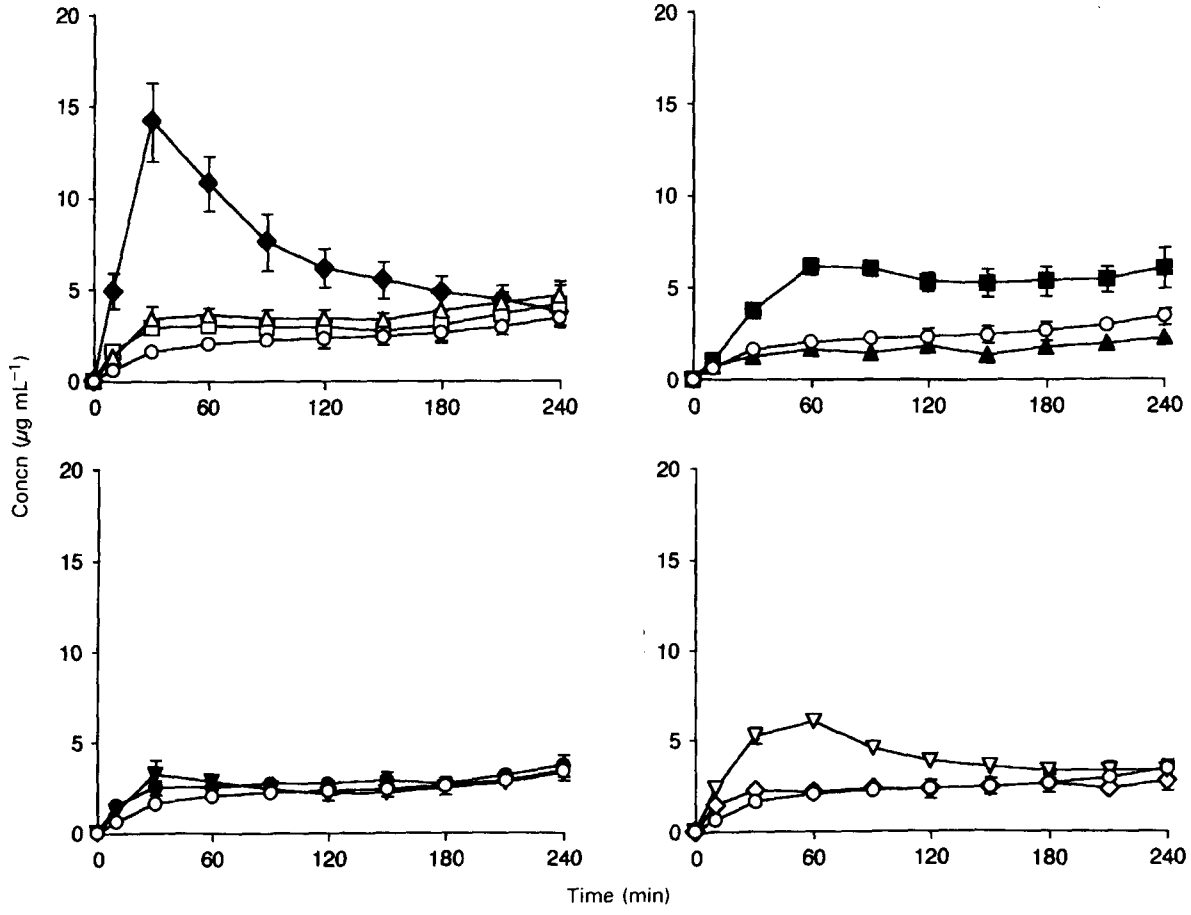

FIG. 1. Effects of various absorption enhancers (20 mM) on plasma concentrations of phenol red after administration to the small intestine. Results are expressed as the mean ± s.e.m. of 4 or 5 experiments. ○ Control, △ sodium glycocholate, □ sodium taurocholate, ● sodium deoxycholate, ● sodium caprate, ▼ diethyl maleate, ■ EDTA, ▲ sodium salicylate, ▽ *N*-lauryl-β-D-maltopyranoside, ◇ mixed micelles.

Table 1. Effects of absorption enhancers on the intestinal absorption of phenol red.

Enhancer	Maximum concentration ($\mu g\,mL^{-1}$)	Time at maximum concentration (min)	Area under plasma concentration-time curve ($\mu g\,min\,mL^{-1}$)	% Absorption
Control	3·39 ± 0·42	216 ± 24·0	534 ± 80·6	35·3 ± 1·87
Sodium glycocholate	4·78 ± 0·43	188 ± 52·5	821 ± 93·0**	50·8 ± 2·33**
Sodium taurocholate	4·51 ± 1·07	156 ± 51·1	704 ± 103**	45·3 ± 1·50**
Sodium deoxycholate	14·1 ± 2·17	30·0 ± 0·0	1660 ± 258	69·0 ± 4·72**
EDTA	6·23 ± 0·63	150 ± 52·0	1190 ± 106	59·4 ± 0·38**
Sodium salicylate	2·30 ± 0·23	216 ± 24·0	363 ± 22·0	43·5 ± 2·30*
Sodium caprate	4·02 ± 0·54	165 ± 49·8	642 ± 40·3	42·1 ± 1·84*
Diethyl maleate	3·87 ± 0·37	135 ± 60·0	594 ± 62·0	34·8 ± 1·02
N-lauryl-β-D-maltopyranoside	5·99 ± 0·40	60·0 ± 0·0	950 ± 63·8**	49·8 ± 2·41**
Linoleic acid polyoxyethylated (60 mol) mixed micelles	3·00 ± 0·44	150 ± 33·5	543 ± 61·3	41·5 ± 3·99

Results are expressed as the mean ± s.e.m. from 4 to 5 rats. *$P < 0.05$, **$P < 0.01$, significantly different from control.

presence or absence of various absorption enhancers. Table 1 summarizes maximum phenol red levels, time to maximum phenol red levels, the area under the plasma concentration-time curve (AUC) and absorption percentages of phenol red. Sodium salicylate, diethyl maleate and the mixed micelles had little effect on the absorption of phenol red. In contrast, a remarkable increase in plasma levels of phenol red was observed after co-administration of phenol red with 20 mM sodium deoxycholate, EDTA or *N*-lauryl-β-D-maltopyranoside, compared with the control (no enhancer); a slight increase in plasma phenol red levels was observed after co-administration of phenol red with sodium glycocholate, sodium taurocholate or sodium caprate. The order of effectiveness ranking of the absorption enhancers in respect of AUC values of phenol red is similar to that observed for its absorption percentages.

Release of protein and phospholipids from the intestinal membrane by absorption enhancers

The effects of absorption enhancers on protein and phospholipid release from the small intestinal membrane are shown in Table 2. Compared with the control (no enhancer), significant quantities of protein were released during perfusion with sodium glycocholate, sodium deoxycholate or EDTA. Slight protein release was observed in the presence of diethyl maleate or sodium caprate. Sodium taurocholate, sodium salicylate and the mixed micelles resulted in protein levels lower than those in the control. Table 2 also lists the amounts of phospholipid released into the lumen of the small intestine during co-perfusion of phenol red with various absorption enhancers. Sodium deoxycholate, EDTA and diethyl maleate caused significant release of phospholipids into the lumen of the small intestine. Sodium glycocholate, EDTA, diethyl maleate, sodium caprate and *N*-lauryl-β-D-maltopyranoside caused minor release of phospholipids. Sodium taurocholate, sodium caprate and the mixed micelles resulted in phospholipid levels similar to those in the control. Of these absorption enhancers, sodium deoxycholate caused the greatest release of tissue protein and phospholipids whereas sodium taurocholate, sodium salicylate and the mixed micelles caused no release of either damage marker into the intestinal lumen.

Table 2. Effects of various absorption enhancers on release of protein and phospholipids release from the small intestinal membrane.

	Protein (mg)	Phospholipid (mg)
Control	11·9 ± 0·92	0·14 ± 0·06
Sodium glycocholate	20·1 ± 2·59*	0·27 ± 0·04n.s.
Sodium taurocholate	9·40 ± 1·19n.s.	0·20 ± 0·07n.s.
Sodium deoxycholate	24·7 ± 3·29**	1·23 ± 0·22**
EDTA	25·1 ± 3·02**	0·56 ± 0·21n.s.
Sodium salicylate	9·94 ± 1·71n.s.	0·16 ± 0·03n.s.
Sodium caprate	16·2 ± 1·20*	0·30 ± 0·12n.s.
Diethyl maleate	15·1 ± 3·56n.s.	0·59 ± 0·15*
N-lauryl-β-D-maltopyranoside	12·2 ± 0·79n.s.	0·28 ± 0·10n.s.
Linoleic acid polyoxyethylated (60 mol) mixed micelles	10·5 ± 1·48n.s.	0·17 ± 0·05n.s.

Results are expressed as the mean ± s.e.m. from 4 or 5 experiments. *$P < 0.05$, **$P < 0.01$, significantly different from control.

Correlation between the promoting effect of various absorption enhancers and their membrane damage

Fig. 2 presents the relationships between protein release and AUC value for phenol red in the presence of various absorption enhancers. Although there is an overall correlation between them, of these absorption enhancers sodium taurocholate and *N*-lauryl-β-D-maltopyranoside seemed to be suitable absorption enhancers because of their low toxicity and good

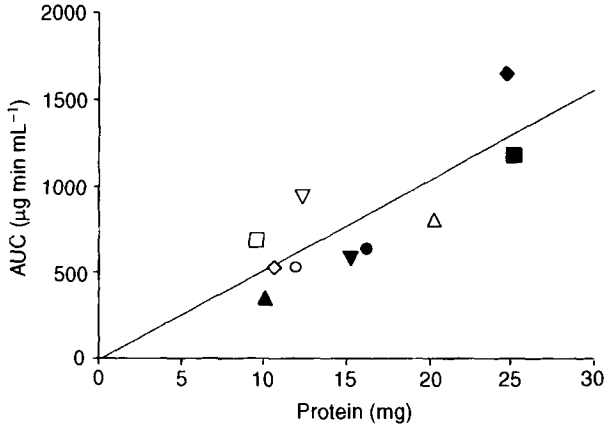

FIG. 2. Correlation between AUC and protein release in the absence or presence of various absorption enhancers (20 mM). Results are expressed as the mean ± s.e.m. of 4 or 5 experiments. ○ Control, △ sodium glycocholate, □ sodium taurocholate, ◆ sodium deoxycholate, ● sodium caprate, ▼ diethyl maleate, ■ EDTA, ▲ sodium salicylate, ▽ *N*-lauryl-β-D-maltopyranoside, ◇ mixed micelles.

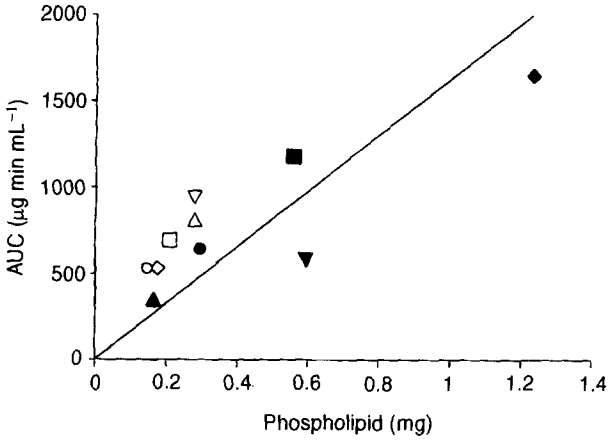

FIG. 3. Correlation between AUC and phospholipid release in the absence or presence of various absorption enhancers (20 mM). Results are expressed as the mean ● s.e.m. of 4 or 5 experiments. ○ Control, △ sodium glycocholate, □ sodium taurocholate, ◆ sodium deoxycholate, ● sodium caprate, ▼ diethyl maleate, ■ EDTA, ▲ sodium salicylate, ▽ *N*-lauryl-β-D-maltopyranoside, ◇ mixed micelles.

absorption-promoting effects. Fig. 3 also shows the correlation between the AUC value for phenol red and the release of phospholipids into the perfusates. It seems that AUC correlates almost exactly with phospholipid release. Fig. 3 shows that *N*-lauryl-β-D-maltopyranoside, sodium taurocholate and sodium glycocholate promoted the absorption of phenol red and had low toxicity.

Discussion

In this study we used an in-situ loop method to investigate the effectiveness of various absorption enhancers and their local toxicity to the small intestinal mucosa. As shown in Table 1, sodium deoxycholate, EDTA and *N*-lauryl-β-D-maltopyranoside significantly enhanced the absorption of phenol red from the small intestine in rats. Sodium deoxycholate and EDTA, however, caused marked damage to the small intestine membrane and are thus not useful for clinical application. Sodium deoxycholate was the most effective absorption enhancer used in this experiment. For the bile salts, the ranking order of promoting effect was sodium deoxycholate > sodium glycocholate > sodium taurocholate. Murakami et al (1984) reported that the effect of deoxycholate on the rectal absorption of sodium ampicillin was more remarkable than that of sodium glycocholate and sodium taurocholate. It was reported that sodium deoxycholate, which caused the most extensive release of protein and phospholipids, was also the most effective of bile salts on nasal and rectal absorption of drugs (Yamamoto et al 1992; Hosoya et al 1994). Our results are consistent with these previous reports. Bile salts have been shown to facilitate peptide and protein absorption from various absorption sites. It was reported that bile salts enhance permeability by removing epithelial cells, which constitute a major permeability barrier (Hersey & Jackson 1987). In addition, Gordon et al (1985) suggested that bile salts might interact with cell membranes to form a reverse micelle which acts as a channel which increases the permeability of the test compounds. These findings suggested, therefore, that bile salts enhance the absorption of phenol red from the intestine by these mechanisms.

It is believed that EDTA increases intestinal mucosal permeability by depleting Ca^{2+} from tight junction areas, thereby opening these normally tight junctions. Cassidy and Tidball (1967) reported that phenol red absorption was greatly increased, whereas tissue levels of Ca^{2+} and Mg^{2+} were depleted by EDTA. They also found that replacement of Ca^{2+} and Mg^{2+} in the perfusion solution restored the normally low permeability of phenol red and normalized the tissue levels of these cations. Yamashita et al (1987) recently examined the effect of EDTA on the electrical parameters of sulphanilic acid permeation through the rat jejunum in-vitro and found that the absorption-promoting effect of EDTA occurred primarily through paracellular pathways. In our study EDTA significantly enhanced the absorption of phenol red with significant release of protein and phospholipids from the small intestinal mucosa. Aungst & Rogers (1988) showed that EDTA enhanced rectal absorption of insulin to the same levels as laureth-9. Morishita et al (1993) reported that the promoting effect of EDTA on intestinal absorption of insulin was more remarkable than that of sodium glycocholate and sodium caprate. Further, EDTA caused more cellular damage to the mucosa compared with sodium caprate or sodium glycocholate (Muranishi 1990). Our results are consistent with these previous reports.

Sodium salicylate, diethyl maleate and the mixed micelles have little or no effect on the absorption of phenol red across the small intestine in rats. Sodium salicylate has no significant absorption-promoting effect for phenol red in the rat small intestinal perfusion model (Swenson et al 1994). In contrast, Aungst & Rogers (1988) reported that sodium salicylate remarkably enhanced insulin absorption across the rectal membrane in rats. The mixed micelles enhanced the absorption of streptomycin in the large intestine, and to a lesser extent in the small intestine (Muranishi 1990). We also suggested that the ranking order of sensitivity to the enhancers is: rectum > colon > small intestine > stomach > skin (Muranishi 1990). From these reports it was suggested that there is a site-dependent enhancing effect of sodium salicylate on drug absorption, and that higher concentrations might be needed for it to enhance the absorption of drugs. This might, in part, explain our results.

Local toxicity has been assessed by measurement of haemolysis, protein release, phospholipid release and by morphological observation. Haemolysis is an easy way to assess local toxicity. Sometimes the results obtained by this method might not be extrapolated directly to the mucosal cells exposed to the enhancers (Lee et al 1991). Although morphological observation has also been widely used to assess local toxicity it cannot be used for quantitative assessment of local toxicity to mucosal membrane; biological markers such as protein and phospholipids release are, on the other hand, suitable for such quantitative analysis and so protein and phospholipid release were used as measures of local toxicity in this experiment.

The correlation between AUC and protein and phospholipid release is presented in Figs 2 and 3, respectively. It was found that *N*-lauryl-β-D-maltopyranoside and sodium taurocholate had comparatively good absorption-promoting effect and low toxicity. A significant enhancement of phenol red absorption was observed in the presence of *N*-lauryl-β-D-maltopyranoside. In addition, *N*-lauryl-β-D-maltopyranoside and sodium taurocholate resulted in phospholipid and protein levels similar to

those in the control. *N*-lauryl-β-D-maltopyranoside, an alkyl-saccharide, has recently been found to reduce surface tension and to have absorption-promoting activity in the gastrointestinal tract. We also showed that rectal absorption of carboxyfluorescein, fluorescein isothiocyanate dextrans and insulin was improved by co-administration of *N*-lauryl-β-D-maltopyranoside (Murakami et al 1992). Ohtani et al (1991) reported that *N*-lauryl-β-D-maltopyranoside is one of the most effective enhancers of pulmonary absorption of fluorescein isothiocyanate dextrans. We also reported that pulmonary absorption of insulin was enhanced in the presence of *N*-lauryl-β-D-maltopyranoside (Yamamoto et al 1994). Swenson et al (1994) showed that sodium taurocholate enhanced the absorption of phenol red across the small intestinal membrane in rats and that taurocholic acid resulted in phospholipid levels similar to those in the control.

It might be concluded that at 20 mM concentrations *N*-lauryl-β-D-maltopyranoside and sodium taurocholate effectively enhance intestinal absorption and have low toxicity.

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