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The effects of Tween 80, Myrj 52 and PEG 8000 upon the rate of pentobarbital disappearance, cumulative water flux and histological changes in the rat small intestine

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Summary

The effect of aqueous solutions of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-6} M concentrations of Tween 80, Myrj 52 and PEG 8000 on the cumulative water flux and rate of disappearance of pentobarbital from the rat small intestine was investigated using the in situ rat gut technique. Water served as the control treatment. Tissue samples of the small intestine were examined microscopically to determine if the histological effects of different solutions (10^{-2} and 10^{-4} M) could be related to the disappearance rate and cumulative water flux data. An inverse relationship between rate of disappearance and concentration was observed. The same relationship was also noted with cumulative water flux and concentration. A general relationship between high rate of disappearance and a high cumulative water flux was also noted. The Tween 80 solutions exhibited a high water flux at all concentrations. The microscopic examination of the tissues revealed that those solutions which caused the most significant disturbances in the mucous layer and the interstitial spaces resulted in the lowest rates and fluxes. The relative order of the differences noted in the histological evaluation were directly related to the differences noted in the rates and fluxes of the different treatments. The Tween 80 solutions had the least effect on the mucous layer and the amount of water in the interstitial spaces. These solutions also exhibited statistically greater ($P < 0.05$) values for the cumulative water flux and rate of drug disappearance from the lumen when compared to the other treatments or to the water controls. The 10^{-2} M solutions of Myrj 52 and PEG 8000 demonstrated significant microscopic changes and low values for the cumulative water flux and rate of pentobarbital disappearance. The Tween 80 solutions demonstrated flux, rate and histological changes that were inconsistent with the data observed in the Myrj 52 and PEG 8000 solutions.

Introduction

Non-ionic surfactants and large molecular weight polyethylene glycols have been shown to affect the bioavailability of orally administered drugs (Chiou and Riegelman, 1970; Gibaldi and Feldman, 1970; Kaur et al., 1979; Walters et al., 1981; Rubinstein et al., 1981). Different concentrations of these vehicles can either enhance or inhibit the absorption from the gastrointestinal tract (Florence and Gillan, 1975a; Kitazawa et al., 1977). Higher concentrations of surfactants generally decrease the absorption rate due to the formation of micelles in the lumen (Yamada and

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Yamamoto, 1965), inhibition of certain enzymes in the glycocalyx or in the enterocyte (Taylor, 1963; Rodgers et al., 1984; Florence and Gillan, 1975b), or by decreasing the rate of fluid transport through the mucosa (Kitazawa et al., 1977). The permeation of the drug or surfactant molecule from the lumen of the small intestine into the enterocyte involves passage through the mucous layer, the glycocalyx and the microvilli. The mucous layer is composed of mucopolysaccharides secreted by the goblet cells and serves as a protective coating. The glycocalyx is a multicomponent and multifunction layer of glycoproteins and enzymes that extends from the surface of the microvilli. The glycocalyx contains lipids, disaccharidases, peptidases, enterokinase, ATPase, alkaline phosphatase and specific glycoproteins which may bind certain substances (Weiss, 1983). This latter characteristic may be responsible for the specific absorption of certain substances at certain sites (i.e. the "window of absorption"). Beneath the glycocalyx are the microvilli of the enterocytes.

The glycoproteins are the macromolecules responsible for the viscous and gel-forming characteristics of the mucous layer. Transport through this layer is dependent upon the configuration or cross-linkage of these various macromolecules (Morris and Rees, 1978). This layer has been characterized as the rate-limiting barrier, or unstirred layer, for the transport and absorption of ergot peptide alkaloids (Franz and Vonderscher, 1981). A characterization of molecular diffusion through this glycoprotein network has been proposed by relating the diffusion coefficient of the drug to the molecular characteristics of the gel (Peppas et al., 1984). Data will be presented demonstrating a relationship between the histological observations of the glycocalyx and enterocytes to the rate of disappearance of pentobarbital and the cumulative water flux from the lumen after exposure to dilute concentrations of Tween 80, Myrj 52 and PEG 8000.

Materials and Methods

Aqueous solutions of Tween 80, Myrj 52 and PEG 8000 at 10^{-2}, 10^{-3}, 10^{-4} and 10^{-6} M concentration were prepared. The Tween 80 and Myrj 52 were obtained from ICI Americas, Wilmington, DE. The PEG 8000 was obtained from J.T. Baker Chemicals, Phillipsburg, NJ. The [^{14}C]pentobarbital and [^{3}H]PEG 4000 were from New England Nuclear, Boston, MA. All reagents were used as supplied. The water control treatment and all solutions were prepared from distilled/deionized water (pH 5.6).

Male Sprague–Dawley rats (210–225 g) were allowed access to a small amount of food 12 h prior to surgery. Water was allowed ab libitum. Anaesthesia was induced and maintained by ether inhalation. Cannulation of 40 cm of the small intestine distal to the bile duct was performed according to the in situ rat gut technique (Doluisio et al., 1969). The intestinal segment was flushed with 50 ml of 37°C saline and returned to the abdomen. The incision was covered with gauze wetted with warm saline. Each experimental group consisted of three rats. The water control group contained six rats. Five ml of each test solution or the water control was added to the intestinal segment. Each solution was spiked with a maximum of 5 μg of [^{14}C]pentobarbital and 1 μg of tritiated PEG 4000. The PEG 4000 served as a non-absorbable marker to measure water flux. Fifty μl samples were drawn from the duodenal syringe at time zero and every 5 min for up to 30 min. The samples were dispersed in 15 ml of scintillation cocktail (Ready-Solv MP, Beckman Instruments, Fullerton, CA). Samples were quantitated in a Beckman LS 7500 Scintillation Counter equipped with automatic quench control. The counting efficiency was greater than 25% for the tritium channel and greater than 85% for the ^{14}C-channel in the dual channel mode.

For the histopathology studies male Sprague–Dawley rats (210–225 g) were anaesthetized and a 40 cm segment of the small intestine was cannulated as previously stated. Lanthanum chloride (Sigma Chemicals, St. Louis, MO) was added to the 10^{-2} and 10^{-4} M solutions of Tween 80, Myrj 52, PEG 8000 and the water controls. The final concentration of lanthanum chloride was 10 mM. The lanthanum chloride was used as an electron dense extracellular marker for electron microscopy (Leeson and Leeson, 1982). In this

study the lanthanum chloride was used to denote any changes in the mucous layer, microvilli or intercellular spaces. Five ml of the test solutions were placed in the intestinal segments as before. After 20 min, the segment was flushed with a fixative containing 0.01 M phosphate buffer at pH 7.2, 1% glutaraldehyde, 4% formaldehyde and 10 mM lanthanum chloride. A 1 cm length of this intestine taken 15 cm from the pyloric sphincter was excised and placed in fresh fixative that did not contain the lanthanum chloride. The rat was then sacrificed. The tissue was placed in fresh fixative overnight. The tissue was then macerated and placed in fresh fixative for 1 h, washed in 0.01 M cacodylate buffer and a 7% sucrose solution (ICN Pharmaceuticals, Covina, CA). A second fixative of 1% aqueous osmium tetroxide (Polysciences, Warrington, PA) in Zetterqvist buffer, pH 7.2, was used to preserve the fine structures and enhance the contrast in the electron microscopy sections. The samples were then stained with 7% uranyl acetate (Ladd Research Ind., Burlington, VT) for 30 min, dehydrated through graded ethanols (70, 95, 100%), and rinsed in propylene oxide (Electron Microscopy Sciences, Ft. Washington, PA). Then the tissues were embedded in a resin (Poly/Bed 812-Araldite 502, Polysciences, Warrington, PA). Tissue sections of approximately 70 nm were sectioned from the hardened resin block using an ultramicrotome. These sections were placed on 200 mesh copper grids for observation and photography under a Phillips 301 electron microscope. Two segments from each of the treatments were prepared in this manner. All samples were submitted in randomly labeled vials for preparation and histological evaluation under blind conditions.

Results

In the in situ studies the rate that the [^{14}C]pentobarbital disappeared from the lumen was first-order over the time course of the study. The activity from the tritiated PEG 4000 was used to calculate the percent water efflux over each 5-min interval according to the equation:

$$\% \text{ Water Flux} = \frac{(\text{dpm})_{t+5} - (\text{dpm})_t}{(\text{dpm})_t} \times 100.$$

The water flux for some treatments was so rapid that the luminal volume was inadequate to collect a 30-min sample. Therefore, the percent cumulative water efflux used statistical comparison was taken over 25 min for all treatments. Table 1

TABLE 1

STUDENT'S *t*-TEST RESULTS COMPARING THE RATE OF PENTOBARBITAL DISAPPEARANCE AND % CUMULATIVE WATER FLUX FOR THE WATER CONTROLS VS EXPERIMENTAL TREATMENTS

Treatment	n	K_{abs} (min^{-1}) Avg. (S.D.)	*P* value	% Cum. Flux Avg. (SD)	*P* value
Water controls	6	0.077 (0.010)	–	71.12 (04.34)	–
PEG 8000 10^{-2} M	3	0.076 (0.008)	> 0.10	51.48 (04.85)	< 0.01
10^{-3} M	3	0.077 (0.007)	> 0.10	66.13 (11.85)	> 0.10
10^{-4} M	3	0.090 (0.004)	< 0.05	86.17 (05.56)	< 0.05
10^{-6} M	3	0.088 (0.004)	< 0.10	83.00 (09.93)	> 0.10
MYRJ 52 10^{-2} M	3	0.059 (0.004)	< 0.05	53.88 (02.32)	< 0.01
10^{-3} M	3	0.078 (0.003)	> 0.10	59.47 (03.45)	< 0.01
10^{-4} M	3	0.094 (0.010)	< 0.10	82.83 (04.46)	< 0.05
10^{-6} M	3	0.108 (0.015)	< 0.10	92.17 (10.84)	< 0.01
TWEEN 80 10^{-2} M	3	0.077 (0.001)	> 0.10	88.74 (01.90)	< 0.01
10^{-3} M	3	0.104 (0.014)	< 0.10	92.58 (05.34)	< 0.01
10^{-4} M	3	0.107 (0.006)	< 0.01	84.93 (03.16)	< 0.01
10^{-6} M	3	0.115 (0.013)	< 0.05	95.96 (10.16)	< 0.10

presents the data from Student's *t*-test comparing the rate of pentobarbital disappearance and the percent cumulative water flux of the water controls with each of the test solutions. The Myrj 52 and PEG 8000 solutions demonstrate similar trends in that concentrations greater than or equal to 10^{-3} M the rates and fluxes are less than or equal to the water controls. At concentrations less than 10^{-3} M they are greater than the water controls. The Tween 80 solutions demonstrated a large percent cumulative flux and high rate of disappearance at all concentrations. It is significant that the most dilute concentration, 10^{-6} M, still resulted in a rate and flux greater than the water controls. An analysis of variance (ANOVA) was performed on the data by within-concentration

TABLE 2

STATISTICAL SUMMARY, ANOVA OF PERCENT CUMULATIVE WATER FLUX AND RATE OF DISAPPEARANCE OF [^{14}C]PENTOBARBITAL

Treatment		Within concentration. Post hoc comparisons *	
		% Cumulative flux	K_{abs} (min^{-1})
Water			
10^{-2} M	PEG 8000	T > W, M, P	no pair-wise contrasts
	Myrj 52	W > M, P	are significant
Water			
10^{-3} M	PEG 8000	T > W, P, M	T > M, P, W
	Myrj 52		
	Tween 80		
Water			
10^{-4} M	PEG 8000	P, T, M > W	T > W
	Myrj 52		
	Tween 80		
Water			
10^{-6} M	PEG 8000	T, M > W	T, M > W
	Myrj 52		
	Tween 80		
		Within treatment. Post hoc comparisons *	
		% Cumulative flux	K_{abs} (min^{-1})
Water			
PEG 8000	10^{-2} M	10^{-4} M, 10^{-6} M, W > 10^{-2} M	($P > 0.050$)
	10^{-3} M		
	10^{-4} M		
	10^{-6} M		
Water			
Myrj 52	10^{-2} M	10^{-6} M > W	10^{-6} M > 10^{-3} M, W, 10^{-2} M
	10^{-3} M	10^{-6} M, 10^{-4} M > 10^{-3} M	10^{-4} M > 10^{-2} M
	10^{-4} M	10^{-6} M, 10^{-4} M, W > 10^{-2} M	
	10^{-6} M		
Water			
Tween 80	10^{-2} M	10^{-6} M, 10^{-4} M, 10^{-3} M, 10^{-2} M > W	10^{-6} M > 10^{-2} M, W
	10^{-3} M		10^{-4} M > 10^{-2} M, W
	10^{-4} M		10^{-3} M > W
	10^{-6} M		

* Post hoc analysis utilized Scheffés Method of multiple comparisons ($P = 0.05$). Actual values for averaged data are presented in Table 1.

and within-treatment comparisons. The water control treatment was included in each group. If the ANOVA indicated a significant difference existed ($P < 0.05$), then a post hoc analysis of the means in the group was undertaken. Scheffe's Method of multiple comparisons was used (Snedecor and Cochran, 1982). Table 2 illustrates the treatments that were significantly different. They also demonstrate which of the means are statistically different within the treatment.

The within-concentration comparison of the different treatments (Table 2) indicate that at higher concentrations the water flux and rate of disappearance were lower than at the more dilute concentrations. An exception to this trend are the Tween 80 solutions. They consistently demonstrated a high water flux or rate of disappearance throughout the concentration range. These solutions demonstrated a statistically significant difference ($P < 0.05$) not only over the other treatments, but also over the water control group.

The same conclusions regarding the effect of dilution on flux and rate can be applied to the within-treatment ANOVA (Table 3). Again, the Tween 80 solutions resulted in fluxes and rates that were generally statistically larger than the water controls ($P < 0.05$).

Table 3 shows the results of the histological evaluations of the tissue segments exposed to the 10^{-2} and 10^{-4} M treatments. Two segments for each treatment were submitted for evaluation under blind conditions. Both light microscopy and transmission electron microscopy were used to examine the tissues. The slides were graded according to the amount of interstitial swelling and the penetration of lanthanum chloride between the microvilli. Zero denotes the presence of small amounts of interstitial water and the presence of lanthanum chloride only on the surface of the microvilli tips. Four denotes the presence of significant amounts of interstitial water and the extensive penetration of lanthanum chloride between the microvilli. The data indicate that the treatments which had the greater amount of lanthanum chloride penetration between the microvilli also showed the largest amount of interstitial swelling. This trend is present whether one observes the data at each concentration or for a particular treatment. There appears to be a pattern associated with the decrease in microvilli length and the increase in the concentration of the Myrj 52 or PEG 8000 treatments as presented in Table 3. The tissues exposed to the Tween 80 solutions showed the least amount of histological change at either concentration. In fact, the water controls demonstrated more interstitial swelling than the Tween 80-treated groups. These observations were reproducible with different fragments within the tissue segment.

Discussion

In observing the effects of either the Tween 80, Myrj 52 or PEG 8000 solutions upon the rate of

TABLE 3

HISTOLOGICAL STUDY

Treatment	AMT of interstitial swelling		Length of microvilli (μm)	Penetration of $LaCl_3$ between microvilli
	Light microscopy	TEM **		
10^{-2} M PEG 8000	4+	4+	1.1	4+
10^{-2} M Myrj 52	1+	2+	1.1	3+
10^{-2} M Tween 80 *	0	0	1.8	0
Water	2+	1+	2.9	trace
10^{-4} M PEG 8000	2+	1+	1.4	2+
10^{-4} M Myrj 52	2+	1+	1.5	1+
10^{-4} Tween 80	0	0	1.5	0

* Some evidence of ischemia in this tissue sample. ** Transmission electron microscopy.

pentobarbital disappearance, percent cumulative water flux, or the histology of the enterocyte, there appear to be at least two systems involved. One in which the concentration inversely affects percent cumulative water flux and rate of drug disappearance. The in situ and microscopic observation data from the Myrj 52 and PEG 8000 groups are in this system. Higher concentrations of surfactant solutions result in numerous factors which could decrease drug absorption; micellularization (Florence and Gillan, 1975a), increased viscosity and physical blocking of the membrane by the surfactant polymers (Yamada and Yamamoto, 1965), increased "water-holding" capacity of the surfactant which may decrease the transmucosal water flux (Kitazawa et al., 1977), increased inhibition of various extra- or intracellular enzyme systems (Taylor, 1963; Marchesi and Palade, 1967), or degradation of the mucous layer or epithelial cells (Bryan et al., 1980; Whitmore et al., 1975).

Many of these mechanisms may be applicable to the Myrj 52 and PEG 8000 data. The 10^{-2} M solution of Myrj 52 was approximately 2.04% (w/v) which is well above its reported critical micelle concentration (cmc) of 0.011% (ICI Americas, Wilmington, DE) so micellularization may have decreased the drug absorption from the higher concentration solutions. According to the data in Table 1, the rate of drug disappearance and water flux only exceeded the water controls when the concentration was at the cmc or lower. The statistical results from Tables 1–3 indicate that the previous statements may also be applied to the PEG solutions. The effects of either treatment on the tissue samples were also very similar. However, at the higher concentration (10^{-2} M) the PEG 8000 resulted in greater penetration of the lanthanum chloride between the microvilli and the appearance of more interstitial water. Similar observations (including shortened villi) were made in a study comparing the histologic damage of PEG 2000 and Myrj 52 solutions on the rat intestinal mucosa (Bryan et al., 1980). The swelling of the interstitial spaces as a consequence of water absorption in the rat intestine has been reported (Tomasini and Dobbins, 1970).

At the higher surfactant concentrations the penetration of the lanthanum chloride between the microvilli is more noticeable in the Myrj 52 and PEG 8000 treatments. The presence of this electron dense marker between the microvilli indicates that the mucous layer has been penetrated and the various glycoproteins and enzymes located in the glycocalyx may have been disturbed. Some of these proteins may have specific binding or recognition functions which would be inhibited or destroyed when the glycocalyx was disrupted. Enzymes and transport systems have been shown to be affected by low surfactant concentrations (lipoprotein lipase, pancreatic lipase, ATPase, glucose uptake and water transport) (Taylor, 1963; Florence and Gillan, 1975b; Marchesi and Palade, 1967). Therefore, the interstitial swelling may be the result of an alteration of the system involved with maintaining fluid equilibrium or transport.

The data collected from the Tween 80 solutions did not coincide with that of the other two groups. All of the Tween 80 dilutions resulted in a larger percent cumulative water flux than the water controls. Three of the four dilutions resulted in a faster rate of drug disappearance, and the fourth (10^{-2} M) was not significantly different from the water controls. Throughout the ANOVA in Table 2 it can be observed that the Tween 80 solutions consistently resulted in rapid fluxes and rates. However, the microscopic evaluation of the tissue resulted in nothing remarkable in the degree of disturbance of the glycocalyx or the presence of unusual amounts of interstitial water.

If it is assumed that the disruption of the glycocalyx can be associated with the swelling of the interstitial spaces, then the treatment which shows the least changes in the glycocalyx should result in the least interstitial swelling. The Tween 80 data demonstrate this trend. However, the water control treatment also demonstrated a lack of microvilli penetration, but it had notable interstitial swelling. Tissues exposed to the Tween 80 solutions had large fluxes and disappearance rates indicating that water transport was increased. Since no interstitial swelling occurred the water must have passed through the tissue. This more efficient water transport may have contributed to its consistently high percent cumulative water flux.

One must be cautious in interpreting the tissue slide data since the conditions depicted in that one

particular slice represent only one moment in time in one area. The changes demonstrated in one section may not be reproducible in the tissue a few centimeters away, nor after a small change in time if these surfactant effects are easily reversible. However, the tissue slices from a specific treatment did demonstrate similar characteristics. Numerous studies have been made utilizing various in vitro, in vivo and in situ models in an attempt to understand the effects of surfactants upon drug absorption. Due to the diverse nature of the drugs, surfactants, and modeling systems, the research has been less than definitive. Studies trying to relate cmc, surface area per molecule, HLB, surface activity or adsorption to some biological activity have not been very indicative (Walters et al., 1982). Investigators have demonstrated that some surfactants can be absorbed (Rodgers et al., 1984; Azmin et al., 1982). Once absorbed they may exert effects on the membrane, the internal functions of the enterocyte, or possible systemic effects (Rodgers et al., 1984; Bryan et al., 1980). Before the drug or surfactant molecule can be absorbed it must pass through a complex collection of mucopolysaccharides, glycoproteins, and enzymes which may enhance or inhibit its transport. Research attempting to model the factors affecting molecular diffusion through this macromolecular gel have been made (Morris and Rees, 1978; Peppas et al., 1969). The results of the present investigation may partially explain some of the effects of surfactants upon this gel. Any disturbances in the gel may affect the inherent recognition or enzyme functions which may be of importance to those involved in the development of drug delivery systems.

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