The Influence of Intestinal Mucus Components on the Diffusion of Drugs

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Purpose. Mucus, a potential diffusional barrier to drug absorption, is a complex mixture of mucin and other components. The objective of this study was to investigate the composition of native pig intestinal mucus (PIM) and the influence of identified mucus components on drug diffusion.

Methods. The mucus components were separated by CsCl-density gradient centrifugation and further analyzed. The self-diffusion coefficients of mannitol, metoprolol, propranolol, hydrocortisone, and testosterone, ranging in lipophilicity from logK = −3.1 to logK = 3.3, were determined, using a small scale tracer technique. The diffusion of drugs in PIM, in solutions or dispersions of individual mucus components, and in an artificial mucus model (MLPD) reconstituted from the major mucus components mucin, lipids, protein, and DNA was compared.

Results. The dry weight of pig intestinal mucus contained (%, w/w); mucin (5%), lipids (37%), proteins (39%), DNA (6%), and unidentified materials. The most commonly occurring lipids were free fatty acids, cholesterol, and phospholipids while the most common protein was serum albumin. In PIM, but not in the purified pig gastric mucin (PPGM) solution, the diffusion of the lipophilic drugs metoprolol, propranolol, hydrocortisone, and testosterone was reduced compared to that of the hydrophilic drug mannitol. The diffusion of the lipophilic drugs was also significantly reduced in a dispersion of identified mucus lipids compared to that of mannitol. The diffusion in MLPD was similar to that in PIM for mannitol, propranolol, hydrocortisone, and testosterone, but somewhat lower for metoprolol.

Conclusions. Lipids, rather than mucin glycoproteins, are a major component which contributes to reduced diffusion of drugs in native intestinal mucus. The results suggest that reconstituted artificial mucus models are interesting alternatives to native mucus models.

KEY WORDS: mucus; drug diffusion; hydrophobic interaction; absorption barrier; drug absorption; intestinal drug transport.

INTRODUCTION

The gastrointestinal mucus layer acts as a barrier to the diffusion and/or absorption of various drugs (1–4). However, it is not known which of the components of mucus are responsible for the barrier properties. In a recent study we found that native pig intestinal mucus (PIM) gave more information regarding the barrier properties of mucus than purified pig gastric (PPGM) since the diffusion of drugs was dependent on charge and lipophilicity in PIM, but not in PPGM (5). PIM contains all the components of a native mucus layer while PPGM comprises mucin molecules and buffer only. It appears, therefore, that components other than mucin molecules influence drug diffusion in the mucus layer. In this study, we have identified the components of PIM and studied their influence on diffusion of drugs with different physico-chemical properties using mannitol as control. The diffusion of drugs in artificial mucus composed of a mixture of the major PIM components was also studied.

MATERIALS AND METHODS

Materials

^{3}H-propranolol (specific activity (s.a.): 21.2 Ci/mmol), ^{14}C-hydrocortisone (s.a.:53.5 mCi/mmol), ^{14}C-mannitol (s.a.: 56.7 mCi/mmol), ^{3}H-testosterone (s.a.: 109.0 Ci/mmol) were obtained from New England Nuclear through DU Medical Scandinavia AB, Stockholm, Sweden. ^{3}H-metoprolol (s.a.: 125 mCi/mmol) and metoprolol were a kind gift from Dr Jan-Erik Löfroth, Astra Hässle AB, Mölndal, Sweden. Soybean phosphatidylcholine (S-PC), cholesterol, and linoleic acid were kindly donated by Dr Anders Carlsson at Scotia LipidTeknik, Stockholm, Sweden. Mucin (type II; partially purified from porcine stomach), pig serum albumin, deoxyribonucleic acid from calf thymus, propranolol, testosterone, hydrocortisone, mannitol, polyoxyethylene sorbitan mono-laureate (Tween 80), and sodium dodecylsulphate (SDS) were obtained from Sigma Chemicals, St Louis, MO, USA. Sodium azide (NaN_3), chloroform (rein Merck), methanol (analytical grade, Merck), and glass beads (0.2 mm diameter) were obtained from Kebo AB, Stockholm, Sweden. 1 ml plastic syringes were obtained from Terumo, Leuven, Belgium. Immunoglobulin G was obtained as Gammanorm™ from Pharmacia and Upjohn, Stockholm, Sweden. SDS-PAGE Phast Gel 10–15, SDS buffer strips and Phastgel Blue R were obtained from Pharmacia, Uppsala, Sweden. Mercaptoethanol was obtained from BIO-RAD, Richmond, CA, USA.

Methods

Identification of Mucus Components

PIM was prepared as described previously (5). Briefly, the intestinal mucosa of freshly slaughtered pigs were gently scraped with a spoon after rinsing of the intestines with cold water. Since the pigs were fasted overnight after a light meal in the evening only small amounts of material was rinsed away. The collected mucus was pooled, immediately frozen in aliquots, and kept at −20°C until further use. The dry weight was determined by lyophilization.

The analysis of mucus components was performed according to published procedures. Briefly, PIM was reduced and alkylated, and the soluble material (98.4%) was subjected to isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride (6). The centrifugation was performed in a Beckman 70-Ti rotor (40,000 rpm, 15°C, 65 hr). The tubes were emptied from the bottom, and fractions (approximately 0.75 ml) were collected and analyzed for absorbance, sialic acid content, and density. The "top" fractions were pooled and used to measure lipid and protein content. Fractions containing

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mucin and DNA were pooled, and subjected to a second density-gradient run in CsCl/0.5 M guanidinium chloride to separate these components. Pooled fractions of each component were lyophilized and weighed. The identity of each component was based on its density (6).

Identification of Lipids. PIM was frozen in a round flask, and the contents lyophilized. The dry material (748mg) was extracted with 10 ml chloroform:methanol in the proportion 2:1 twice, 1:1 once, and 1:2 twice. The pooled portions of chloroform:methanol and the residues of the extraction were placed under reduced pressure in a rotary evaporator until dry. The lipids were analyzed as follows. High pressure liquid chromatography (HPLC) was used to analyze neutral lipid classes (Herslöf, B. G. and Tingvall, P., personal communication) and polar lipid classes (7). Identification of components was made by comparing retention times with those of authentic standards. Fatty acid composition was determined using gas chromatography (GC) on lipid samples after hydrolysis and methylation (8,9). The presence of glycolipids was analyzed according to methods used by Slomiany and co-workers (10).

Identification of Proteins. The protein content of unfractionated PIM was compared with that of the protein fraction from the density gradient centrifugation using gel electrophoresis. The stability of the proteins in PIM was investigated by incubating PIM at 37°C in the presence and absence of protease inhibitors for 50 h. Samples were reduced in boiling 5% mercaptoethanol and 2.5% SDS in 10 mMTris/HCl buffer, and subjected to SDS-PAGE using the Pharmacia PhastGel System (10%–15% gradient gel), Separation Technique File No. 110. The gels were stained with Coomassie blue and photographed.

Model Drugs

The diffusion of mannitol (used as control because its diffusion appears to be unaffected by the various media chosen (5)) and two homologous pairs of drug molecules metoprolol and propranolol (which carry a net positive charge at physiological pH) and testosterone and hydrocortisone (which are uncharged) were determined. The diffusion of positively charged drugs may be reduced in mucus due to ionic interactions with the negatively charged mucin molecules. The stability of the drugs was analyzed using chromatographic methods (5).

Determination of Self-Diffusion Coefficients

The self-diffusion coefficients of mannitol (octanol/water distribution ratio (logK) = −3.10), metoprolol (logK = −1.34), propranolol (logK = 0.59), hydrocortisone (logK = 1.61), and testosterone (logK = 3.32) were determined. LogK and pKa-values were obtained from the literature (11,12). Whenever applicable, logK values at pH 6.5 (mean pH of diffusion media) were calculated according to: $\log K = \log P - \log(1 + 10^{pKa-pH})$ (13). A tracer technique (14) was used to determine the self-diffusion coefficients in the various diffusion media as described previously (5). Briefly, 1 ml plastic syringes with their tips cut off were filled with diffusion medium containing non-radioactive drug together with small glass beads (0.2 mm diameter). A 0.2 µl aliquot of radio-labeled drug solution, with the same concentration of drug as in the diffusion medium, was added to the diffusion medium at the tip of the syringe. The diffusion experiments were terminated after 20–50 h incubation in 37°C by pressing out the contents of the syringe and cutting this into 10 pieces which were subsequently analyzed for radioactivity. The determination of the diffusion coefficients was based on a weighted least square fit of the normalized theoretically determined radioactivity values to the normalized experimental radioactivity values in each piece (5) according to:

$$\text{theor } nN_i = \text{erf}((x_i + l_i)/\sqrt{4 \cdot D_s \cdot t}) - \text{erf}(x_i/\sqrt{4 \cdot D_s \cdot t})$$

where i denotes one piece of the content of a syringe, numbered 1–10, theor nN_i is the normalized theoretically determined radioactivity for piece number i, x_i is the lower limit of the distance of piece number i in cm from the application site (the top of the syringe), t is the duration of the experiment, and D_s is the diffusion coefficient (the only variable). The curve was fitted to the equation using a computer program developed inhouse. The diffusion coefficients were multiplied by the molecular weight$^{1/3}$ of the diffusing substance in order to compensate for the influence of the variable molecular weights of the drug molecules on the diffusion coefficients according to the Stokes-Einstein equation.

Diffusion Media

The choice of diffusion media for drug diffusion studies was based on the results from density gradient centrifugation, analysis of lipids and proteins, and comparison with the literature on the luminal contents of the human gastrointestinal tract.

Purified pig gastric mucin (which is commercially available) was used as a model for mucin (5). A lipid mixture of 82% linoleic acid, 12% cholesterol, and 6% S-PC was used as a model for the lipids present in native intestinal mucus. Pig serum albumin was chosen as a model protein since a major band corresponding to the molecular mass of albumin was detected in the SDS-PAGE gel. IgG, (which, in contrast to IgA, is readily available) was used as a model for secretory IgA (sIgA) which is secreted into the gastrointestinal lumen as part of the immune system. Mammalian DNA from calf thymus was also used in the diffusion media.

The pH of each diffusion medium was determined prior to dispensing into the diffusion chambers. The pHs ranged between 6.4 and 6.9, except for the lipid-containing diffusion media and the artificial mucus which had pHs of 5.8.

Preparation of Diffusion Media

A reference diffusion medium containing 8% (w/w) PIM was chosen since it was difficult to pack the syringes with undiluted PIM (14% to 17%, w/w) due to its high viscosity (5). After identification and quantification of the components of mucus the corresponding concentrations of the individual components in 8% PIM were calculated. Thus, the following diffusion media were prepared (%; w/w): 0.4% PPGM; 3.0% of the lipid mixture described above (LIP); two different model proteins: 3.1% pig serum albumin (PSA) and 3.1% immunoglobulin G (IgG); and 0.5% (DNA). Artificial mucus, MLPD, containing 0.4% PPGM, 3.0% LIP, 3.1% PSA, 0.5% DNA and 0.75% Tween 80 was also prepared. The diffusion of the drugs in phosphate buffer (PB) was also determined. All diffusion media contained 0.04% sodium azide to prevent bacterial growth, 10 mM phosphate buffer and non-radioactive drug at a concentration corresponding to that of 20 000 dpm/0.2 µl

of the radio-labeled drug. PIM was thawed and diluted with phosphate buffer to 8% (w/w). PPGM was prepared as described previously (5). Briefly, lyophilized purified pig gastric mucin was dissolved in 6M guanidinium hydrochloride, dialyzed and frozen. Before the experiments, PPGM was thawed and diluted with buffer. LIP was prepared by thorough mixing of a lipid mixture containing the most abundant lipids in PIM (linoleic acid, cholesterol and S-PC) with Tween 80 and buffer by connecting two syringes with a piece of silicone tubing and pressing the contents back and forth ten times. Tween 80 was included at the lipid:surfactant molar ratio of 3:1 in order to obtain a lipid dispersion that was physically stable during packing of the diffusion chambers. Control experiments with Tween 80 in buffer (same concentration as in LIP and MLPD) showed no difference in diffusion compared to PB alone with testosterone. The size of the lipid particles was determined 30 min after preparation using a laser diffraction instrument (Coulter LS230, Coulter Corporation, Miami, FL, USA). The mean volume diameter, d_v, of the lipid particles in LIP was 12.0 ± 10.5 μm. Pig serum albumin, IgG and DNA were dissolved in phosphate buffer at appropriate concentrations. MLPD was prepared by mixing purified pig gastric mucin, lipids, pig serum albumin, DNA and Tween 80 at the concentrations mentioned above in the same manner as LIP.

Statistics

Results are presented as mean values ± standard deviations (s.d.). The unpaired Student's t-test, adjusted with the Bonferoni method, was used to test the significance of the difference between the control mean value and the measured mean values.

RESULTS

Identification of Mucus Components

PIM was separated by density gradient centrifugation and 87% (w/w) of the dry weight of PIM was identified. The two major components of PIM were lipids and proteins while mucin glycoproteins were present in lower amounts (5%, w/w), Table I. Of the unidentified material 1.6% (w/w) was insoluble material not included in the density gradient run and 7.7% (w/w) from fractions of lower density than mucin which may have contained unglycosylated mucins.

Table I. Components of PIM[a]

Component	% (w/w) of dry weight
mucin	5
lipids	37
proteins	39
DNA	6
other	13

[a] The contents were analysed by weighing the lyophilised fractions obtained after density gradient centrifugation, see materials and methods.

Table II. Composition of Neutral Lipids in PIM[a]

Neutral lipids	% of total lipids
cholesterol ester	0.1
cholesterol	12.0
ceramide	0.7
free fatty acids:	
palmitic acid	17.7
stearic acid	13.0
oleic acid	10.6
linoleic acid	24.2
other free fatty acids	16.6

[a] Extraction and analysis of lipids were performed as described in materials and methods.

Identification and Stability of Lipids

The lipid mixture obtained after extraction of lyophilized PIM was found to consist mainly of free fatty acids with cholesterol as the second largest component, Tables II and III. Subsequent analysis showed that the four major fatty acids were linoleic acid, palmitic acid, stearic acid, and oleic acid, Table II. None of the other 16 fatty acids identified amounted to more than 5% of the fatty acids. The lipids contained 5% polar lipids, the most abundant being lyso-phosphatidylcholine, Table III. The concentration of glycolipids in the lipid mixture was less than 5% (data not shown).

Identification of Proteins

Gel electrophoresis of unfractionated PIM revealed a pronounced band at 67 kDa, corresponding to the molecular mass of serum albumin. Comparable band patterns were obtained for unfractionated PIM, the protein fraction obtained from density gradient centrifugation, and samples incubated with and without protease inhibitors.

Drug Diffusion Experiments

The diffusion of the 4 lipophilic model drugs was greatly reduced in PIM compared to that of the hydrophilic control drug mannitol, whereas diffusion in PPGM was similar to that

Table III. Composition of Polar Lipids in PIM[a]

Polar lipids	% of total lipids
phosphatidylethanolamine	0.9
phosphatidylcholine	0.8
sphingomyelin	0.7
lyso-phosphatidylcholine	2.7

[a] Extraction and analysis of lipids were performed as described in materials and methods.

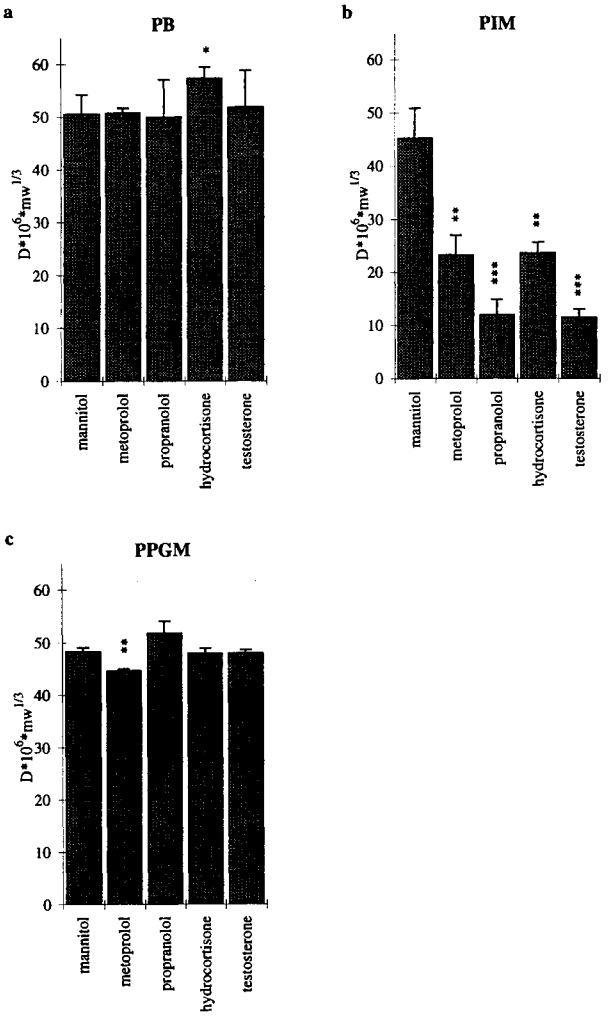

Fig. 1. Diffusion coefficients * $mw^{1/3}$ of the model drugs in a) PB, b) 8%, w/w PIM and c) 0.4%, w/w PPGM. Height of bars indicates mean ± s.d., n = 4–27. Significantly different from the diffusion of mannitol: *p < 0.05, **p < 0.01, and ***p < 0.001.

of mannitol, figure 1. The diffusion of the model drugs was also greatly reduced in LIP compared to that of mannitol, figure 2. The diffusion of hydrocortisone and testosterone in PSA was greatly reduced compared with that of mannitol, figure 2 while the diffusion of metoprolol and propranolol was only slightly reduced in this medium. Only small reductions in the diffusion of model drugs relative to mannitol were observed in DNA, and diffusion in IgG was similar for all 5 drugs. Comparable diffusion rates were obtained in MLPD, PIM, and LIP for all drugs studied except for metoprolol which had reduced diffusion in LIP and MLPD compared with that in PIM, figure 3.

DISCUSSION

In this investigation, we found that native pig intestinal mucus contains a large proportion of lipids and proteins. The lipid component had the greatest influence on the diffusion of a series of drugs. When reconstituting an artificial mucus from the diffusion media used as models of the individual mucus components, diffusion results similar to those in PIM were obtained, suggesting that artificial mucus compositions are interesting alternatives to more complete, native mucus models such as PIM.

Identification of Mucus Components

The dry weight of undiluted PIM ranged from 14% to 17% (w/w), comparable to the 16.2% reported for rat native intestinal mucus (15). In PIM, mucin constituted approximately 0.7% which falls within the range of 0.5–5% obtained for the mucin content of purified mucus gels (16). The large amounts of other components than mucin is also in agreement with previous studies on rat (15) and pig (17) small intestinal mucus.

To our knowledge, this is the first investigation of the lipid classes in small intestinal mucus. Several studies have shown that the mucus layer in the stomach contains lipids which act as a hydrophobic barrier against luminal acid (10,18,19). The major lipids in PIM were identified as fatty acids, cholesterol, and lyso-PC. The predominant phospholipid of both pig gastric mucosa and mucus has been shown to be PC (20). The main

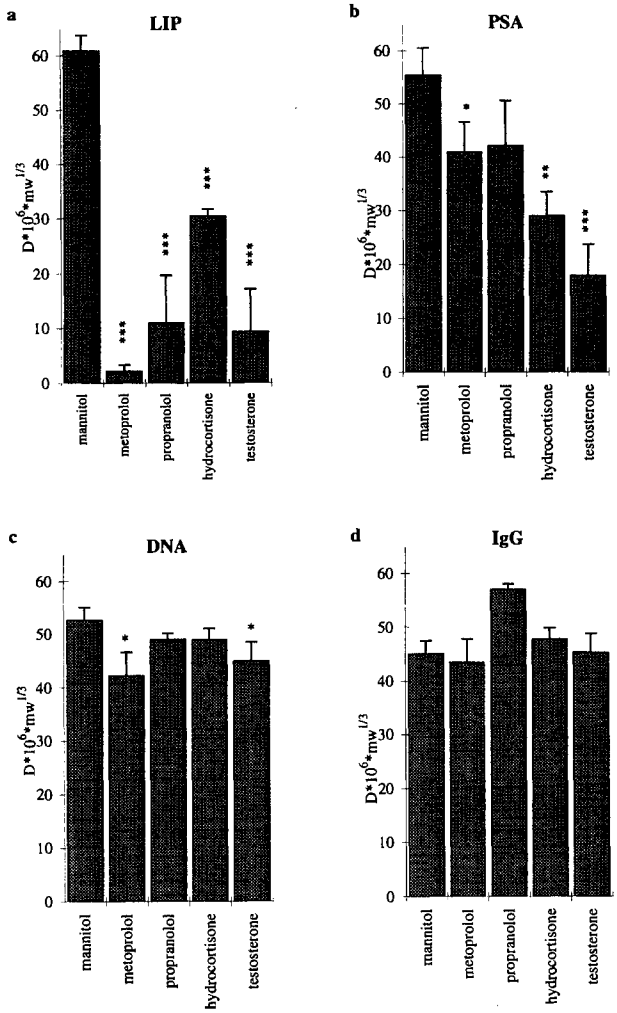

Fig. 2. Diffusion coefficients * $mw^{1/3}$ of the model drugs in solutions of the individual components of native intestinal mucus, a) 3.0% LIP b) 3.1% PSA c) 0.5% DNA d) 3.1% IgG, all w/w. Height of bars indicates mean ± s.d., n = 3–4. Significantly different from the diffusion of mannitol: *p < 0.05, **p < 0.01, and ***p < 0.001.

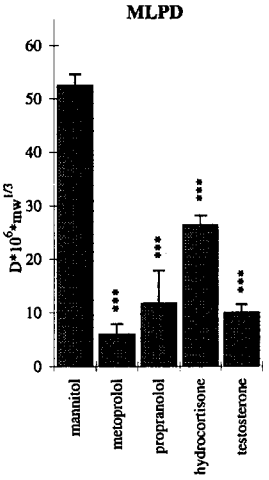

Fig. 3. Diffusion coefficients * $mw^{1/3}$ of the model drugs in an artificial mucus solution containing 0.4% PPGM, 3.0% LIP, 3.1% PSA and 0.5% DNA, all w/w. Height of bars indicates mean ± s.d., n = 4. Significantly different from the diffusion of mannitol: ***$p < 0.001$.

component of the cell membranes of sloughed cells are phospholipids such as PC and cholesterol (21). The main dietary lipids are digested into free fatty acids and lyso-PC (22) which explains the large amounts of these lipids in PIM.

SDS-PAGE revealed that PIM contained more than 15 different proteins. The most pronounced band on SDS-PAGE corresponded to the molecular mass of albumin. Endogenous proteins, including albumin, are secreted in saliva, gastric juice, bile, and pancreatic secretions (23). Also, appreciable quantities of serum proteins, such as albumin, pass into the gastric mucus (24) and the intestinal fluid (25). Thus, the serum albumin found in PIM most likely originated from any or all of these sources. Secretory IgA is secreted into the gastrointestinal tract, in addition to the proteins mentioned above (25,26), with an estimated production as large as 3000 mg/day in humans (27). Therefore, separate diffusion studies of drugs in an immunoglobulin solution were performed. PAGE-gels with similar band pattern were obtained after incubation of PIM in the presence and absence of protease inhibitors at 37°C for 50h.

The detected DNA most probably originated from the sloughed intestinal cells. Diffusion studies in DNA were motivated since DNA also is an important component of mucus, especially in mucus in cystic fibrosis patients, where it contributes to an increased viscoelasticity of the sputum (28).

The lipids, proteins, and DNA may either be considered as an integral part of the mucus layer or as impurities of the mucus preparation (24,29). However, epithelial cells, which are sloughed from the tips of the villi in large amounts, are trapped in the mucus layer, and digested food from the stomach may be mixed with the mucus. It is well established that digested food is an integral part of the mucus layer. Therefore, studies on fed rather than fasted animals will more correctly resemble the normal situation in vivo both in pigs and in man. Thus, irrespective of the origin of these mucus components, they are present in the small intestinal lumen as a part of the milieu that diffusing drug molecules have to encounter.

Drug Diffusion Experiments

Density gradient centrifugation yielded only small amounts of each of the mucus components. Therefore, model diffusion media were chosen for each mucus component for the investigation of the influence of these components on drug diffusion.

In agreement with our previous study (5), the reduced diffusion of lipophilic drugs in PIM, relative to in PPGM, suggests that some component other than mucin may be responsible. This hypothesis was supported by the reduced diffusion of lipophilic drugs in LIP, indicating that the drugs interacted with or partitioned into the lipids. The diffusion of hydrocortisone was reduced in the lipid-containing diffusion media less than anticipated from its octanol/water distribution ratio, suggesting that also other factors such as hydrogen-bonding capacity (which is not well described by the octanol/water distribution ratio (30)) contribute to the interaction between drugs and mucus. The finding, that the effect of the lipid component remained in MLPD, provided further support for this hypothesis. Thus, a strong linear correlation between drug diffusion in LIP and MLPD was observed ($r^2 = 0.99$). The results suggest that LIP is the only component influencing drug diffusion in PIM. However, the diffusion studies with PSA suggest that protein binding also could be of importance. Further investigations are needed to clarify this issue since no additive effect of PSA was observed in MLPD. The results of this study indicate that mucin and DNA do not interact with hydrophilic and lipophilic low molecular weight drugs. It is more likely that these gel-forming and viscosity increasing agents are more significant barriers to the diffusion of larger drugs such as peptides and proteins (5).

The diffusion results obtained in MLPD were similar but not identical to those obtained in PIM, suggesting that it may be difficult to reconstitute the complete structure of native mucus. A possible loss of the gel-forming properties of PPGM, and a suboptimal mixing of especially the lipid components of MLPD, may have contributed to this result. Further studies are therefore needed to confirm the utility of artificial mucus models in drug absorption studies.

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