ELSEVIER

International Journal of Pharmaceutics 134 (1996) 15–25

international journal of pharmaceutics

Drug binding to gastric mucus glycoproteins

Pavan G. Bhat, Douglas R. Flanagan, Maureen D. Donovan*

Division of Pharmaceutics, College of Pharmacy, The University of Iowa, Iowa City, IA 52242, USA

Received 2 March 1995; revised 30 August 1995; accepted 27 September 1995

Abstract

Absorption of drugs across any mucosal tissue may involve interactions with the mucus gel overlying the tissue. Drug binding to the mucus glycoproteins, in particular, can reduce the amount of free drug available for absorption. In order to evaluate the extent of drug binding to mucin, a purified model mucus system containing primarily the large glycoprotein fraction (400 kDa) of gastric mucus was developed for use in drug binding studies. The extent of binding of six selected compounds (albuterol, rifampicin, *p*-amino-salicylic acid, isoniazid, pyrazinamide, and pentamidine) to mucus glycoproteins was studied. The binding of each drug to a model plasma protein, bovine serum albumin (BSA), was also investigated. Binding studies were performed by diafiltration, which combines characteristics of equilibrium dialysis and ultrafiltration in a continuous system. All the compounds selected showed affinities of the same order of magnitude to mucin despite being chemically dissimilar and exhibiting differing ionization states. This suggests that binding to gastric mucus glycoproteins is non-specific in nature with similar types of binding forces involved in the binding of all the compounds tested. Results also showed that the drug–protein association constants for BSA and mucin were of the same order of magnitude only for drugs with low binding affinities. When the binding constants to BSA were moderate to high, the corresponding drug binding constants to mucin were lower by at least one order of magnitude. Based on these results, it can be concluded that the binding behavior of drugs to gastric mucin is non-specific in nature with binding constants of a low magnitude. BSA cannot be used to estimate binding to mucin, especially when the drug exhibits moderate to high affinity for BSA.

Keywords: Mucus; Mucin; Drug binding; Diafiltration; Glycoprotein; Albuterol; *p*-Amino-salicylic acid; Isoniazid; Pyrazinamide; Pentamidine; Rifampicin

1. Introduction

Interactions between drugs and proteins are of widespread interest since virtually every drug interacts with some kind of protein (i.e., cell associated carriers during absorption, cell associated protein receptors, or during its metabolism and removal from the body). Binding of the drug to proteins other than the target sites can reduce the effectiveness of the drug. Binding to glycoproteins in mucus may limit the bioavailability of drugs administered orally, intranasally or by inhalation.

The molecular units of the mucus glycoproteins (mucins) interpenetrate to form a linked macroscopic network which is responsible for the viscoelastic and gel-forming properties of mucus

* Corresponding author.

SSDI 0378-5173(95)04333-6

solutions (Gu et al., 1988). Mucus is found at all internal mucosal surfaces directly or indirectly in contact with the external environment where it plays an important role in the maintenance of water balance, cytoprotection, lubrication and removal of cellular debris. In previous studies characterizing the interaction of drugs with mucus solutions, Saggers and Lawson (1966) reported that tetracyclines bind to porcine gastric mucin; Braybrooks et al. (1975) found that the intestinal absorption of tetracyclines was decreased by ~ 50% in the presence of a mucin dispersion; and Block and Lamy (1969) reported that macromolecules, including gastric mucin, decreased drug absorption in in vitro systems. Nibuchi et al. (1986) studied the interaction of 13 ß-lactam antibiotics and three amino-glycosides with the soluble and the insoluble fractions of mucus. They found differing extents of interactions of the antibiotics with the two different fractions of mucus and concluded that drug interactions with mucin reduce the intestinal absorption of cephaloridine and gentamicin. (Vasseur et al., 1978, 1979) and Tsuchiya et al. (1983) have also reported that surfactants deplete mucin from the intestinal or rectal surfaces. Subsequent increases in drug absorption indicated that interaction of drugs with mucus can affect their bioavailability. Matthes et al. (1992a, 1992b) studied the in vitro interaction of 10 compounds, both polar and non-polar, with native pig duodenal mucus as the first step in developing a model for the intestinal absorption of compounds. They also found that the presence of mucus decreased the rate of drug absorption.

In an attempt to identify the role that drug binding to mucus glycoproteins plays in reducing bioavailability, the extent of binding of six compounds, albuterol, rifampicin, *p*-amino-salicylic acid, isoniazid, pyrazinamide, and pentamidine, to gastric mucus glycoproteins was studied using an in vitro diafiltration technique. The success of albuterol as an aerosolized anti-asthmatic agent is well established. Thus, minimal interaction between albuterol and mucus solution was anticipated. The selection of the other model compounds was made on the basis of their therapeutic application for pulmonary delivery. The compounds selected possess a wide range of physicochemical properties and degrees of plasma protein binding, making them quite useful to test the robustness of this experimental technique to identify compounds whose mucin binding properties might limit their effectiveness. The anti-tubercular compounds were selected since the lungs are a primary site of their therapeutic action. Moreover, anti-tubercular drugs are used clinically with increasing frequency, and most of their pharmacokinetic and binding properties have been well characterized. Pentamidine was studied because of its inclusion in aerosols for AIDS therapy.

Drug binding to gastric mucin was compared to binding to a model plasma protein, bovine serum albumin (BSA). This was done to provide a basis upon which to compare the magnitude of the binding constants obtained to the "percent-bound" measurements typically reported for plasma protein binding. While the majority of the drugs with significant plasma protein binding are bound to albumin, the binding parameters of these drugs to individual plasma proteins are not frequently reported. Moreover, since both the proteins included in this study, mucin and BSA, are polyanionic at physiological pH, investigating the extent of binding to each protein provides evidence regarding the importance of ionic interactions in the binding of the drug compounds.

Diafiltration is continuous ultrafiltration, hence it combines the capabilities of ultrafiltration and equilibrium dialysis in a continuous system. Diafiltration decreases the experimental time required for drug binding studies when compared with equilibrium dialysis. This reduces the potential for drug and protein degradation and concomitant changes in drug binding parameters over long experimental time periods (Reiland, 1981). During a diafiltration experiment, drug solution of concentration C_R is placed in a reservoir which is connected to the ultrafiltration chamber (Fig. 1). A fixed volume of protein solution (V_0) of concentration C_p is placed in the ultrafiltration chamber. Diafiltration begins when the system is pressurized by an external inert gas and drug reservoir solution flows into the ultrafiltration chamber where it interacts with the protein. Any free or unbound drug passes continuously

through the ultrafiltration chamber. Free drug concentrations (C) in the diafiltrate fractions are analyzed and the amount of bound drug calculated. Equilibrium is achieved rapidly with this system and can be considered to be a series of continuous equilibrium dialysis experiments. Thus, it is possible to generate an entire drug-binding isotherm with a single diafiltration run. This reduces variability in the fitted binding parameters and the need to conduct multiple studies to generate sufficient data for a complete binding isotherm.

The utility of diafiltration as a technique to measure drug binding has been demonstrated by Reiland (1981). Binding studies of sodium warfarin, sodium salicylate, sodium benzoate, and *m*- and *p*-hydroxybenzoic acids to BSA were conducted by diafiltration. These studies demonstrated that the binding coefficients obtained by diafiltration compared well to reported values obtained by equilibrium dialysis or ultrafiltration. Diafiltration was also used to characterize drug displacement by compounds which exhibit significant binding.

2. Experimental

2.1. Materials and methods

Sodium *p*-amino-salicylate (PAS), isonicotinic acid hydrazide (INH), pyrazinamide, rifampicin,

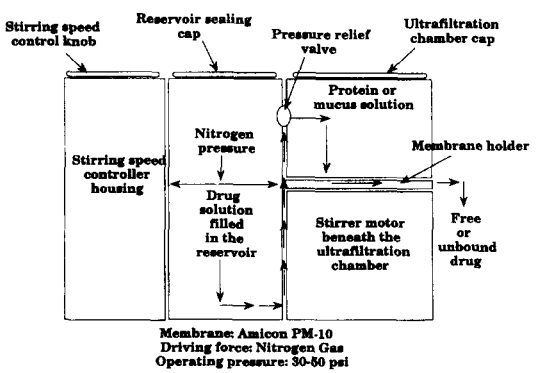

Fig. 1. Schematic of the diafiltration system used in drug-binding studies.

and sodium azide were obtained from Sigma Chemical Co. (St. Louis, MO). Pentamidine isoethionate was obtained from the Walter Reed Army Institute of Research (Washington, DC). Bovine serum albumin (BSA, Fraction V) and pig gastric mucin (Type II, lyophilized) were also obtained from Sigma Chemical Co. (St. Louis, MO). BSA was used without further purification, while the gastric mucus was purified as described below. Distilled deionized water was used in the preparation of all drug and buffer solutions. The buffer system used in all of the binding studies was Sørenson's phosphate buffer, 0.067 M, pH 7.4. Buffers were prepared from monobasic sodium phosphate and dibasic sodium phosphate, heptahydrate (EM Science, Gibbstown, NJ). The Amicon® 8-MC diafiltration unit and Amicon® PM membranes were obtained from Amicon Corp. (Beverly, MA). Dialysis membranes were obtained from Spectrum. (Houston, TX).

2.2. Reconstitution and purification of mucin

Mucus solutions were prepared by a modification of the procedure of List et al. (1978). Pig gastric mucin (40 mg/ml) was suspended in isotonic phosphate buffer, pH 7.4, containing 0.02% (w/v) sodium azide, and stirred at 4°C for 24 h. The suspension was centrifuged at 27 000 × g for 15 min to remove any insoluble material and the supernate was removed. A second centrifugation (27 000 × g for 15 min) was carried out on the supernate, followed by dialysis of the final supernate through a standard cellulose acetate dialysis membrane (MWCO 10–12 000) against several changes of pH 7.4 hypertonic phosphate buffer (0.1 M). The supernate was then dialyzed against several changes of isotonic Sørenson's phosphate buffer, pH 7.4, to re-equilibrate to physiological conditions. The dialysate was centrifuged at 27 000 × g for 15 min and finally ultrafiltered through an Amicon® PM-30 membrane (MWCO 30 000). This insured the elimination of most UV-absorbing low molecular weight peptide contaminants. The mucin solutions were frozen and stored at −20°C until further use (Matthes et al., 1992 a,b).

2.3. Analysis of reconstituted mucus solutions

The molecular weight distribution of each batch of reconstituted and purified gastric mucus solution was analyzed by reverse phase, size-exclusion, high performance liquid chromatography (SE-HPLC) using a Spherogel® TSK 3000 SW, 10 μm, 7.5 mm × 30 cm column (Beckman Instruments, Fullerton, CA). The SE-HPLC column was attached to a matching guard column (Spherogel® guard, 10 μm, 7.5 mm × 7.5 cm). The HPLC system consisted of a solvent delivery system (Model LC-600, Shimadzu USA, Columbia, MD), a variable wavelength detector (Model SPD-6A, Shimadzu USA, Columbia, MD), an auto-injector (SIL-9A Shimadzu USA, Columbia, MD) and an integrator (Model C-R601, Chromatopac, Shimadzu USA, Columbia, MD). The mobile phase consisted of pH 5.0, 0.1 M phosphate buffer containing 0.5 M NaCl flowing at a rate of 0.5 ml/min at room temperature. Protein standards (molecular weight range 1.65–670 kDa) were used to calibrate the column. The standards used were Vitamin B-12 (1.65 kDa), equine myoglobin (17 kDa), chicken ovalbumin (44 kDa), bovine gamma globulin (158 kDa), and thyroglobulin (670 kDa). Standards were obtained from BIORAD Corp. (Hercules, CA). The reconstituted and purified mucus solutions were analyzed under the same conditions as the standards.

Each reconstituted and purified batch of mucus solution was also analyzed gravimetrically for solids content. A fixed volume of mucus solution was weighed into a tared vial and then dried under vacuum at 30°C until a constant weight was obtained.

2.4. Drug-binding studies

Drug-binding studies were performed by diafiltration at room temperature using an Amicon® Micro-Ultrafiltration System, Model 8-MC (Amicon Corp., Beverly, MA) (Fig. 1). The diafiltration system consisted of a speed control housing assembly which powered a stirrer motor and kept the contents of the ultrafiltration chamber uniformly mixed. The drug solution was filled into a reservoir chamber and 4–8 ml of a protein solution (4% BSA or 2.85% mucus) was placed into the ultrafiltration chamber. Amicon® PM-10 membranes (MWCO 10 000) were used in all of the binding studies to retain protein molecules and drug–protein complexes within the ultrafiltration chamber. Amicon® PM membranes are composite in nature, with a polymeric membrane attached to an open spongy cellulosic support. The diafiltration system was connected to an external nitrogen gas source. On pressurizing the system, free drug from the reservoir was forced into the ultrafiltration chamber where it interacted with the protein solution. The 'free' drug exited the ultrafiltration chamber with the diafiltrate fluid and was collected in 2–4 ml aliquots. The aliquots were assayed for free drug by UV spectroscopy with a diode array spectrophotometer (Model 8450A, Hewlett-Packard Company, Palo Alto, CA). After the binding run was completed, the volume of the protein solution in the ultrafiltration chamber was measured by withdrawing the protein solution into a 10 ml disposable syringe. This recovered volume was used to correct for any volume changes in the ultrafiltration chamber over the course of the diafiltration run. The average of the final recovered volume and the initial volume was used in the calculation of drug-binding parameters. All binding experiments were performed at pressures of 30–50 psi to maintain membrane integrity while minimizing experimental run times.

Non-specific drug binding to the Amicon® PM-10 membrane and/or the diafiltration cell was investigated by filling isotonic phosphate buffer (pH 7.4), 8 ml, into the ultrafiltration chamber instead of a protein solution. A diafiltration run was conducted under these conditions to determine whether any drug was lost due to membrane or apparatus binding.

Non-specific mucus glycoprotein binding to the Amicon® PM-10 membrane and/or the diafiltration cell was also investigated by measuring the decrease in protein concentration during a diafiltration experiment. The amount of mucin was measured by the Alcian blue binding method (Hall et al., 1980). The cationic dye, Alcian blue, binds to the polyanionic mucus glycoproteins with resulting precipitation of the dye–glycoprotein

complex and a concomitant decrease in the absorbance of Alcian blue in the supernate. Alcian blue (0.1% (w/v)) at pH 5.8 was prepared in a 0.1 M acetate buffer containing 25 mM magnesium chloride. The solution was centrifuged at $3000 \times g$ to remove any undissolved materials. Supernatant dye solution (1 ml) was added to 3 ml of mucus solution and allowed to equilibrate for 21 h. The reaction mixtures were centrifuged at $3000 \times g$ for 20 min to separate the mucin–dye complex and the supernatant solutions were analyzed spectrophotometrically using a UV diode array spectrophotometer (Model 8450A, Hewlett Packard, Palo Alto, CA).

3. Results and discussion

3.1. Analysis of reconstituted mucus solutions

The molecular weight distribution analysis of the reconstituted gastric mucus solutions showed three distinct molecular weight fractions – a high molecular weight fraction ($\sim 4 \times 10^5$ Da) present in low concentration and two low molecular weight fractions (~ 1800 and ~ 4000 Da) present in much higher concentrations. After purification, the SE-HPLC analysis of the mucus solution showed a mono-disperse distribution of the high molecular weight fraction. The glycoprotein molecular weight obtained ($\sim 4 \times 10^5$ Da) correspond well to the molecular weight of mucin reported previously by several investigators (Lindstedt et al., 1965; Allen and Snary, 1972; Allen, 1978; Allen et al., 1982). While there is significant disagreement over the absolute molecular weight of mucin, there is agreement that the mucin obtained by the reconstitution of lyophilized mucin contains subunits of native mucin, many of which may have been formed during lyophilization of the native mucus solution or by breakdown of the native structure by glycosidases. The low molecular weight fractions obtained following the reconstitution of the lyophilized pig gastric mucin were removed by the dialysis and ultrafiltration steps during purification. This was confirmed by SE-HPLC analysis of the final purified mucus solution. Gravimetric analysis of reconstituted and purified mucus solutions showed the solids content of all the batches to be $2.85 \pm 0.1\%$ w/v. Mucin concentrations in solution were calculated before and after a diafiltration experiment and the amount of mucus glycoproteins lost due to adsorption or non-specific protein binding to the Amicon membranes and/or the diafiltration cell was found to be approximately 3.5% of the original concentration.

3.2. Drug binding studies by diafiltration

3.2.1. Non-specific binding

Drug concentrations in the diafiltrate fractions with buffer present in the ultrafiltration chamber were compared to the drug concentrations predicted by Eq. (1):

$$C_n = C_r(1 - e^{-(V_n/V_0)}) \quad (1)$$

where C_n is the drug diafiltrate concentration in fraction n, C_r the reservoir drug concentration, V_n the cumulative midpoint volume of fractions collected to fraction n, and V_0 the buffer solution volume in the ultrafiltration chamber.

Eq. (1) represents the zero binding case (Reiland, 1981) which predicts the drug diafiltrate concentration in cases where there is no protein solution in the ultrafiltration chamber. It predicts an asymptotic rise in the drug diafiltrate concentration until the concentration is finally equal to the drug reservoir concentration. Any difference between the measured concentration and the concentration predicted by Eq. (1) is attributed to non-specific binding of the drug to the membrane and/or the system components.

Of the drugs studied, only rifampicin showed significant binding to the membrane or components of the diafiltration cell. To limit the influence of irreversible binding on the protein binding results for rifampicin, the membranes were presaturated in rifampicin solution by soaking and agitating them in an incubator shaker (Lab-Line Instruments Inc., Melrose Park, IL) for approximately 12 h at 37°C. The membranes were then thoroughly washed with isotonic buffer solution until excess drug was removed from the membrane. This was followed by immersing the membrane in isotonic buffer solution and shaking

it in an incubator shaker. This process was repeated by changing the buffer until no more rifampicin desorbed from the membrane. These membranes were then used for rifampicin protein binding studies.

3.2.2. Drug–protein binding

Four percent BSA solution, the albumin concentration in plasma, and 2.85% mucus solution, the concentration of mucus obtained by the reconstitution purification and equilibration procedure, were used throughout these experiments. On a molar concentration basis, the BSA solution was approximately 0.6 mM while mucin was approximately 0.07 mM, thus the BSA solution was ~8.4 times more concentrated than the mucin solution.

Diafiltrate drug concentrations were measured, and the amount of drug bound to BSA or to mucin was calculated from the resulting binding curve utilizing the following equation (Reiland, 1981):

$$B = (V_n \times C_r) - (V_0 \times C_n) - A \quad (2)$$

where B is the amount of drug bound, V_0 the protein solution volume in the ultrafiltration chamber, and A the total area under the binding curve (C vs V_n) up to V_n.

Knowing the amounts of free drug and calculating the amount of bound drug using Eq. (2), binding constants were obtained by fitting the amounts of free and bound drug to a Scatchard single site model (Eq. (3)) using a nonlinear, iterative, curve fitting program (ENZFIT™ Elsevier, Amsterdam, The Netherlands) (Upadrashta, 1988)

$$\frac{C_b}{C_p} = \frac{KC}{1+KC} \quad (3)$$

where C is the concentration of unbound drug, C_b the concentration of bound drug, C_p the total protein concentration, and K the drug–protein association constant.

The robustness of these parameters was confirmed by simulating diafiltration curves for the cumulative midpoint volumes using Eq. (4) (Reiland, 1981) and a plotting program (Kaleidagraph®, Synergy Software, Reading, PA)

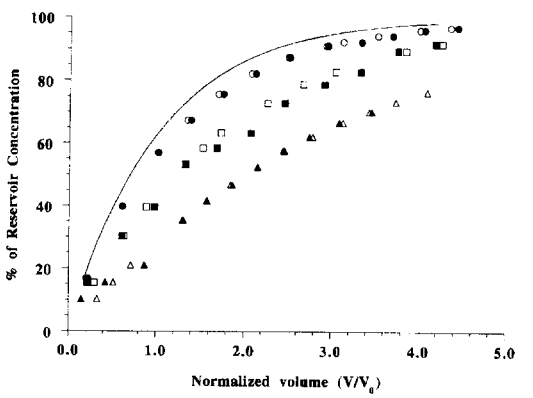

Fig. 2. Plot of percent of reservoir concentration versus cumulative midpoint diafiltrate volume for albuterol (□,■), pentamidine (△,▲) and pyrazinamide (○,●) obtained for binding to 2.85% mucus solution. Solid symbols are experimental data and open symbols are calculated values using the fitted binding constants. The zero binding case is shown by the smooth curve (——).

$$\ln\left(\frac{C_r}{C_r - C}\right)\left[1 + \frac{nKC_p}{(1+KC_r)^2}\right] + \frac{nKC_p}{(1+KC_r)}\left[\frac{KC}{1+KC} + \frac{\ln(1+KC)}{(1+KC_r)}\right] = \frac{V}{V_0} \quad (4)$$

Drug diafiltration curves, with their simulations for binding to each protein, are shown in Figs. 2 to 5. In each of these figures, the data

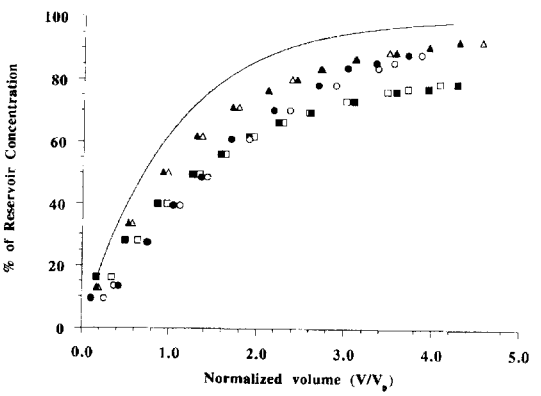

Fig. 3. Plot of percent of reservoir concentration versus cumulative midpoint diafiltrate volume for PAS (△,▲), INH (□,■) and rifampicin (○,●) obtained for binding to 2.85% mucus solution. Solid symbols are experimental data and open symbols are calculated values using the fitted binding constants. The zero binding case is shown by the smooth curve (——).

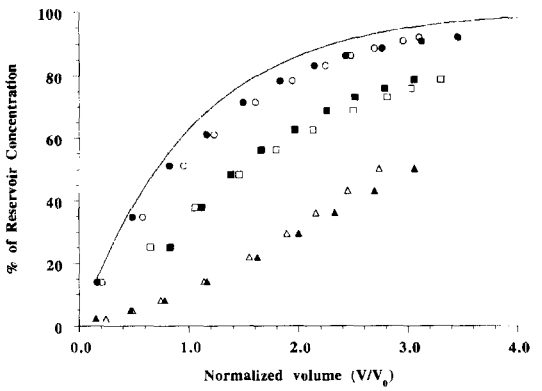

Fig. 4. Plot of percent of reservoir concentration versus cumulative midpoint diafiltrate volume for albuterol (□,■), INH (△,▲) and pyrazinamide (○,●) obtained for binding to 4% BSA solution. Solid symbols are experimental data and open symbols are calculated values using the fitted binding constants. The zero binding case is shown by the smooth curve (——).

from a single diafiltration run are compared to the simulated profile generated from the equilibrium constant obtained from the average value found by fitting Eq. (3) to three or more data sets.

The abscissa in Figs. 2–5 is the normalized cumulative midpoint volume rather than the cumulative volume because the diafiltrate drug concentration better represents the average concentration of the volume fraction than the concentration at the end of the collection interval (Reiland, 1981). The volumes were normalized with respect to the volume of the protein solution in the ultrafiltration chamber (V_0). This factors out the dependence of each curve on the volume of the protein solution in the ultrafiltration chamber. The ordinate in the plots is percent free concentration, which normalizes the effect of the reservoir drug concentration on the diafiltration curves.

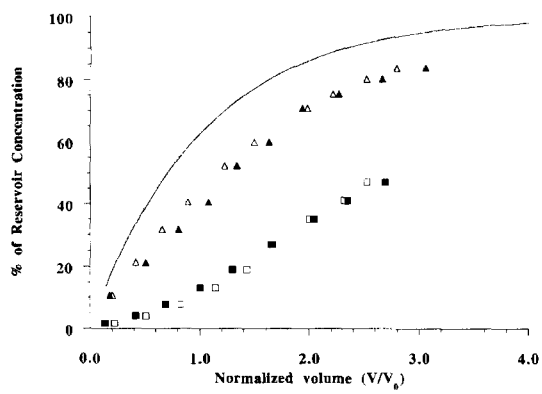

Fig. 5. Plot of percent of reservoir concentration versus cumulative midpoint diafiltrate volume for PAS (□,■) and pentamidine (△,▲) obtained for binding to 4% BSA solution. Solid symbols are experimental data and open symbols are calculated values using the fitted binding constants. The zero binding case is shown by the smooth curve (——).

These figures demonstrate that, for compounds with large association constants, larger cumulative volumes of diafiltrate are required for the free concentration to reach that of the reservoir (C_r). As would be expected for compounds that are highly bound, larger amounts of drug are required to saturate the binding sites on the protein molecules. Larger amounts of drug would also be required to saturate the protein binding sites when there is a larger volume and/or higher concentration of protein solution in the ultrafiltration chamber due to the larger capacity constant of the protein for the drug.

The magnitude of the shift of the experimental diafiltration curves from the zero binding case can be influenced directly or indirectly by the drug–protein association constant, the drug reservoir concentration, the drug reservoir volume, and the concentration of the protein solution in the ultrafiltration chamber. Drugs with high binding constants and high capacity constants would therefore be expected to show the greatest shifts.

It should also be noted that Figs. 2 and 3, combined with Figs. 4 and 5, respectively, cannot be directly compared since the plots represent the binding of drugs to two different proteins, gastric mucin and BSA, respectively. Moreover, since the BSA solution used was ~ 8.4 times more concentrated than the mucin solution, it would be necessary to account for the increased binding capacity of BSA for drugs as compared to mucin solution.

As can be seen from Table 1, the gastric mucin binding constants are all of the same order of magnitude (910–3600 M^{-1}). For BSA, the association constants vary over nearly two orders of magnitude (740–19 000 M^{-1}). For albuterol, pyrazinamide, and pentamidine, all of which show

Table 1
Drug association constants to bovine serum albumin (BSA) and mucin obtained using a Scatchard single-site model

Drug	Reservoir conc. (C_r, mM)	BSA[a] K (M^{-1})	Mucin[a] K (M^{-1})
Albuterol	0.48	2200 ± 100	1700 ± 80
INH	0.73	19 000 ± 1900	3000 ± 20
PAS	0.60	18 000 ± 2100	1200 ± 20
Pentamidine	0.29	1400 ± 20	3600 ± 120
Pyrazinamide	0.81	740 ± 20	910 ± 20
Rifampicin	0.11		1800 ± 30

[a]Average ± SD.

low affinity to BSA, the association constants are of the same order of magnitude for BSA and mucin. However, for the moderate to high binding compounds (PAS, INH, and rifampicin), the association constants for binding to mucin are at least one order of magnitude lower than those for BSA. Statistical analysis of the predictability of mucin binding constants from BSA binding constants indicated that BSA binding constants were not predictive of gastric mucin binding constants ($p < 0.05$). This was true even for drugs with low binding affinities when the binding constants were of the same order of magnitude.

The diafiltration results obtained suggest that rifampicin binds to more than one site on BSA. The experimental data did not fit well to the Scatchard single site model as there was considerable deviation between the experimental and fitted data. The binding curve resembled the curves obtained for INH and PAS binding to BSA (Figs. 4 and 5), although the rise in the percent free concentration was much slower, indicating a higher affinity for BSA. To derive an equation similar to Eq. (4) for two sites was beyond the goal of this work.

At pH 7.4, pyrazinamide and INH are unionized, amino-salicylic acid is anionic, pentamidine is cationic, and rifampicin is zwitterionic. The sialic acid residues on the mucin glycoproteins have a pK_a of 2.6 (Johnson and Rainsford, 1972), thus at pH 7.4 they are completely deprotonated. These residues, along with the ionized sulfate residues on mucin, result in an overall negatively charged biopolymer at pH 7.4. Serum albumin has an isoelectric pH of 5.3; at pH 7.4, albumin has a net negative charge of -17 (Johnson and Rainsford, 1972; Meyer and Silberberg, 1978). Since both of these proteins are negatively charged under physiological conditions, one may suspect that drug association constants to these proteins would be similar if ionic interactions predominate. The difference in the affinities of the drugs to the two proteins indicates that other interactive forces, in addition to ionic interactions, are involved in binding. The other forces which may be involved are hydrogen bonding and hydrophobic interactions. These forces predominate in the binding of other compounds to BSA (Reiland, 1981). For example, albumin has a relatively high affinity for anions in spite of its net negative charge at pH 7.4. This can be explained by intramolecular hydrogen bonding between the hydroxyl and carboxyl groups of the amino acids, resulting in unassociated cationic nitrogen groups available for binding anions (Reiland, 1981). This type of interaction may account for the moderate to high affinity of anionic PAS and zwitterionic rifampicin to BSA. Pentamidine, even though it is cationic, has a low association constant to BSA. This may be due to steric hindrance of the bulky pentamidine molecule preventing access to the ionic binding sites on albumin.

While the compounds selected for investigation in these studies were chemically dissimilar, they all show an affinity on the same order of magnitude to mucin, and do not span a large range of affinities as seen for BSA. This suggests that binding to gastric mucus glycoproteins is non-specific in nature and that similar binding forces may be involved in the binding of compounds to mucin

regardless of their ionic state. Hydrophobic interactions may, at first, seem unlikely since glycoproteins are water soluble and extensively hydrated. However, part of the glycoprotein structure contains a globular protein region (Pearson et al., 1981) which lends a lipophilic character to the glycoprotein structure and results in drugs binding via hydrophobic interactions. In addition, gastric mucus glycoproteins have been shown to be esterified with long chain fatty acids (Slomiany et al., 1983). These lipophilic regions associated with the mucus glycoprotein make hydrophobic associations with drugs a likely possibility. Several other investigators have drawn similar conclusions from their work. Kearney and Marriott (1987) attributed the binding of tetracycline to mucin to a combination of electrostatic forces coupled with hydrophobic binding throughout the pH range of 1 to 11. Matthes et al. (1992a, 1992b) studied the binding of polar and non-polar compounds to pig intestinal mucus and found that they were bound by a non-specific interaction. The observed lack of pH-dependence on the binding of drugs to mucus did not indicate the presence of strong electrostatic interactions, but rather favored drug distribution to hydrophobic areas within mucus. In contrast, Nibuchi et al. (1986) studied the interaction of ß-lactam and aminoglycoside antibiotics with the soluble and insoluble components of rat intestinal mucin and concluded that binding depended on the state of ionization and decreased with increasing ionic strength of the solution.

The dissimilarity in affinities of the compounds studied for BSA and mucin can be easily explained by the difference in composition and conformation of the two proteins. Large differences in amino acid composition alter the binding affinity as well as the binding capacity of proteins. Albumin is a globular protein with approximately 580 amino acid residues (Peters, 1975). Moreover, the conformation of mucin and BSA in solution are also quite different. Mucus glycoproteins, on the other hand, contain a significant number of carbohydrate residues, as many as 160–200 chains per mucin molecule. The carbohydrate composition is polydisperse with respect to the oligosaccharide composition, sequence, and chain length (Carlson, 1966; Oates et al., 1974; Roseman et al., 1968; Spiro, 1970). These carbohydrate chains form a dense, hydrophilic cover for the protein backbone (Forstner et al., 1977; Allen et al., 1974). As a result, considerable amounts of water tend to be immobilized within the fibrillar network of mucin (Creeth et al., 1974), and the equivalent hydrodynamic volumes of mucin molecules are quite high (Allen et al., 1974; Forstner et al., 1977). There have been several models proposed for the secondary and tertiary structure of mucus glycoproteins in solution: the windmill model (Allen and Snary, 1972; Allen et al., 1974), the bottle brush model, the beaded chain model (Robinson and Monsey, 1975), and the flexible thread model (Forstner et al., 1973). In contrast, the secondary structure of albumin consists of 50–55% α-helix and 15% ß-pleated sheet conformation with the remaining residues being arranged as a random coil (Peters, 1975). These significant differences in the conformations of the proteins in solution clearly have a major influence on drug binding.

4. Conclusions

Poelma et al. (1990, 1989) and Komiya et al. (1980) have indicated that the rate-limiting step for absorption of very lipophilic solutes is their transfer across the aqueous diffusion barrier adjacent to the intestinal wall. A portion of this diffusional resistance lies in the mucus layer, but it is difficult to determine the exact contribution of mucus glycoproteins to the overall barrier. This study examined drug–mucin binding to characterize the extent and nature of these interactions. It was found that all of the compounds selected show a binding affinity to gastric mucin which was on the same order of magnitude despite being chemically dissimilar. This suggests that binding to gastric mucus glycoproteins is non-specific in nature, and the forces involved in the binding of compounds to mucin are hydrophobic and Van der Waal's interactions and not due to electrostatic interactions. The low magnitudes of the association constants suggests that mucin-binding alone may not be responsible for poor bioavailabilities through mucosal membranes.

Drug binding to mucin was also compared to binding to a model plasma protein, BSA, in order to compare the order of magnitude of the drug–mucin interaction to that of albumin. Comparisons were also made to assess whether the extent of plasma protein binding had any correlation with drug binding to mucin. Even though the two proteins are both highly anionic at physiological pH, the drug–protein association constants were of similar magnitudes only for drugs showing low binding affinity for both proteins.

Since most of the binding constants to mucin are quite low for the compounds studied, other factors are likely responsible for reductions in permeability through mucus which could decrease drug bioavailability. Such additional factors have also been studied with this reconstituted gastric mucus system and results have shown ~56–90% reductions in the permeability of these compounds through mucus solutions compared to buffer (Bhat et al., 1995). With such information, improved dosage regimens accounting for any drug lost to binding and decreases in permeability could be identified so that drug bioavailability can be optimized.

References

Allen, A., The structure of gastrointestinal mucus glycoproteins and the viscous and gel-forming properties of mucus. *Br. Med. Bull.*, 34 (1978) 28–33.

Allen, A., Bell, A., Mantle, M. and Pearson, J.P., The structure and physiology of gastrointestinal mucus. In Chantler, E.N., Elder, J B. and Elstein, M. (Eds), *Mucus in Health and Disease II*, Advances in Experimental Medicine and Biology, Vol. 144, Plenum Press, New York, 1982, pp. 115–133.

Allen, A., Pain, R. and Snary, D., Formation of mucus gel from the high molecular weight mucoprotein of gastric mucus. *Discuss. Faraday Chem. Soc.*, 57 (1974) 210–220.

Allen, A. and Snary, D., The structure and function of gastric mucus. *Gut*, 13 (1972) 666–672.

Bhat, P.G., Flanagan, D.R. and Donovan, M.D., The limiting role of mucus in drug absorption: Drug permeation through mucus solution. *Int. J. Pharm.*, 126 (1995) 179–187.

Block and Lamy, 1969Reference mentioned in text but not included in reference list.

Braybrooks, M.P., Barry, B.W. and Abbs, E.T., The effect of mucin on the bioavailability of tetracycline from the gastrointestinal tract: in vitro–in vivo correlations. *J. Pharm. Pharmacol.*, 27 (1975) 508–515.

Carlson, D.M., Oligosaccharides isolated from pig submaxillary mucins. *J. Biol. Chem.*, 241 (1966) 2984–2986.

Creeth, J.M., Bhaskar, K.R., Donald, A.S.R. and Morgan, W.T.J., The macromolecular properties of blood-group specific glycoproteins. *Biochem. J.*, 143 (1974) 159–170.

Forstner, J.F., Jabbal, I. and Forstner, G., Goblet cell mucin of rat small intestine. Chemical and physical characterization. *Can. J. Biochem.*, 51 (1973) 1154–1166.

Forstner, J.F., Jabbal, I., Findlay, B. and Forstner, G.G., Interactions of mucins with Ca^{++}, H^{+} ions and albumin, in mucus secretions in cystic fibrosis. In Forstner, J.F. (Ed.), *Mod. Probl. Pediat.*, Vol. 19, Karger, Basel, 1977, pp. 54–65.

Gu, J.M., Robinson, J.R. and Leung, S.H.S., Binding of polymers to mucin/epithelial surfaces: structure–property relationship. *CRC Crit. Rev. Ther. Drug Carrier Systems*, 5(1) (1988) 21–67.

Hall, R.L., Miller, R.J., Peatfield, A.C., Richardson, P.S., Williams, I. and Lampert, I., A colorimetric assay for mucus glycoproteins using Alcian Blue. *Biochem. Soc. Trans.*, 8 (1980) 72.

Johnson, P.M. and Rainsford, K.D., The physical properties of mucins: Preliminary observations on the sedimentation behavior of porcine gastric mucin. *Biochim. Biophys. Acta*, 286 (1972) 72–78.

Kearney, P. and Marriott, C., The effect of mucus glycoproteins on the bioavailability of tetracycline II: Binding. *Int. J. Pharm.*, 35 (1987) 211–217.

Komiya, I., Park, J.Y., Kamani, A., Ho, N.F.H. and Higuchi, W.I., Quantitative mechanistic studies in simultaneous fluid flow and intestinal absorption using steroids as model solutes. *Int. J. Pharm.*, 4 (1980) 249–262.

Lindstedt, C., Lindstedt, S. and Gustafsson, B., Mucus in intestinal contents of germ-free rats. *J. Exp. Med.*, 121 (1965) 201–213.

List, S., Findlay, B.P., Forstner, G.G. and Forstner, J.F., Enhancement of the viscosity of mucin by serum albumin. *Biochem. J.*, 175 (1978) 565–571.

Matthes, I., Nimmerfall, F. and Sucker, H., Mucusmodelle zur untersuchung von intestinalen absorptionsmechanismen I. *Pharmazie*, 47 (1992) 505–515.

Matthes, I., Nimmerfall, F. and Sucker, H., Mucusmodelle zur untersuchung von intestinalen absorptionsmechanismen II. *Pharmazie*, 47 (1992) 609–613.

Meyer, F.A. and Silberberg, A., Structure and function of mucus. In Nugent J. and O'Connor M. (Eds), *Respiratory Tract Mucus*, Ciba Foundation Symposium, Elsevier, Amsterdam, 1978, pp. 203–211.

Nibuchi, J.J., Aramaki, Y. and Tsuchiya, S., Binding of antibiotics to rat intestinal mucin. *Int. J. Pharm.*, 30 (1986) 181–187.

Oates, M.D.G., Rosbottom, A.C. and Schrager, I., Further investigations into the structure of human gastric mucin. The structural configuration of the oligosaccharide chains. *Carbohydr. Res.*, 34 (1974) 115–137.

Pearson, J.P., Allen, A. and Parry, S., A 70,000 molecular weight protein isolated from purified pig gastric mucus glycoprotein by reduction of disulfide bridges and its implication in the polymeric structure. *Biochem. J.*, 197 (1981) 155–162.

Peters, Jr., T., In Putnam, F.W. (Ed.), *The Plasma Proteins*, Vol. I, Academic Press, New York, 1975, 141 pp.

Poelma, F.G.J., Breas, R. and Tukker, J.J., Intestinal absorption of drugs. IV. The influence of taurocholate and L-cysteine on the barrier function of mucus. *Int. J. Pharm.*, 64 (1990) 161–169.

Poelma, F.G.J., Tukker, J.J. and Crommelin, D.J.A., Intestinal absorption of drugs. I. The influence of taurocholate on the absorption of dantrolene in the small intestine of the rat. *J. Pharm. Sci.*, 78 (1989) 483–490.

Reiland, T.L., Diafiltration studies of drug–protein binding and displacement. Ph.D. Thesis, The University of Iowa, 1981.

Robinson, D.S. and Monsey, J.B., The composition and proposed subunit structure of egg-white ß-ovomucin. *Biochem. J.*, 147 (1975) 55–62.

Roseman, S., Biosynthesis of glycoproteins, gangliosides and related substances. In *4th Int. Conf. on Cystic Fibrosis of the Pancreas (Mucoviscidosis)*, part 11, Karger, Basel, 1968, pp. 244–269.

Saggers, B.A. and Lawson, D., Some observations on the penetration of antibiotics through mucus *in vitro*. *J. Clin. Pathol.*, 19 (1966) 313–317.

Slomiany, A., Slomiany, B., Witas, H. and Newman, L.J., Isolation of fatty acids covalently bound to the gastric mucus glycoprotein of normal and cystic fibrosis patients. *Biochem. Biophys. Res. Commun.*, 113 (1983) 286–293.

Spiro, R.G., Glycoproteins. *Annu. Rev. Biochem.*, 39 (1970) 559–638.

Tsuchiya, S., Aramaki, Y., Ozawa, S. and Matsumaru, H., Effect of polyoxyethylene cetyl ether on the absorption of 3',4'-dideoxykanamycin B from rat rectum. *Int. J. Pharm.*, 14 (1983) 279–290.

Vasseur, M., Ferand, G. and Pousse, A., Do low doses of deoxycholate modify the release of rat jejunal brush border hydrolases? *Pflugers Arch. Ges. Physiol.*, 379 (1979) 297–299.

Vasseur, M., Ferand, G. and Pousse, A., Rat intestinal brush border enzyme release by deoxycholate in vivo. *Pflugers Arch. Ges. Physiol.*, 373 (1978) 133–138.

Upadrashta, S., Solution-state decomposition and protein binding of anthralin. Ph.D. Thesis, The University of Iowa, 1988.