Competitive Inhibition of Drug–Protein Interaction in Eye Fluids and Tissues

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Abstract □ Binding of drugs to proteins in fluids and tissues of the eye previously was shown to modify significantly the bioavailability of a number of drugs. This adverse effect is particularly important in the precorneal portion and anterior chamber of the eye, since tears and aqueous humor are turned over at relatively rapid rates, removing both free and bound forms of the drug. In addition, rapid drainage of instilled solutions also removes substantial drug quantities. The drug-protein interaction can be minimized through the use of competitive inhibitors of protein binding. These agents occupy binding sites normally bound by drug, increasing free drug concentration. *In vitro* experiments with eye fluids and tissues, as well as *in vivo* studies in rabbits, support the concept of competitive inhibition as a means of significantly improving drug bioavailability. These experiments are principally concerned with competitive inhibition in the precorneal portion of the eye. Pilocarpine nitrate, a miotic drug with low binding affinity for albumin, shows a 10-fold increase in biological activity in the presence of the competitive inhibitor cetylpyridinium chloride. This increase in response is suggested to be due, in part, to competitive inhibition of drug–protein interaction. Competitive inhibition of drug binding in tears and the precorneal portion of the eye can be applied successfully without the attendent problems when the concept is applied to blood proteins. Tear proteins have apparently less substrate carrier function as compared to blood proteins, there is less total protein in tears as compared to blood, and the amount of drug plus inhibitor applied to the eye is small. Thus, side effects due to undesired substrate displacement are not present when rather nonspecific competitive inhibitors are used in ophthalmic systems. Extrapolation of the pilocarpine nitrate data to drugs with higher protein binding affinities suggests that substantial increases in drug activity can be expected through competitive inhibition of drug–protein interactions in eye fluids and tissues.

Keyphrases □ Drug-protein interactions in eye fluids and tissues—effect of competitive protein binding inhibitors on drug availability □ Protein binding—effect of competitive inhibitors on drug availability, eye fluids and tissues □ Ophthalmic bioavailability—effect of competitive protein binding inhibitors on drug-protein interactions in eye fluids and tissues □ Bioavailability, ophthalmic—effect of competitive protein binding inhibitors on drug-protein interactions in eye fluids and tissues

An earlier report (1) from this laboratory described the importance of drug binding to proteins in tissues and fluids of the eye. Tears, cornea, and, in some circumstances, aqueous humor contain appreciable quantities of protein, which bind drugs and reduce their effective free concentrations. Since both tears and aqueous humor are turned over at relatively rapid rates, removing both free and bound forms of the drug, the influence of drug–protein binding on drug bioavailability is significant. The present report is concerned with a proposed method for competitive inhibition of drug–protein interaction through the use of rather nonspecific "biologically inert" inhibitors. A substantial increase in drug activity in the presence of these inhibitors is observed and offers an attractive method to improve drug bioavailability in the eye.

The concept of competitive inhibition of drug–protein binding to increase free drug concentration is not new (2). Synergistic behavior of drug effects is considered to be in some instances a result of competitive displacement of a strongly bound drug (2). Suggestions of the use of substrates for intentional competitive inhibition have been made (3), but apparently no specific application has been reported. There are several reasons why systemic application of competitive inhibition to increase drug activity has not been successful: (*a*) blood proteins normally serve as carriers for numerous substrates, so a competitive inhibitor must be highly specific for sites on the protein or displacement of normal substrates will occur followed by undesirable side effects; and (*b*) the enormous amount of protein present in blood requires that large amounts of the inhibitor be used to cause successful competitive inhibition. Thus, application of competitive inhibition of drug–protein binding in the blood is very limited, and no successful application of the concept to an *in vivo* situation could be found.

However, when the proposal is considered with respect to the eye, it has much greater potential. The relative isolation of the eye coupled with the limited protein content and the lack of extensive carrier function of this protein, as compared to blood protein, makes the concept of competitive inhibition of drug–protein interaction an attractive, workable proposal for increasing drug bioavailability (4). This report is concerned with the feasibility of competitive inhibition of drug–protein binding in eye fluids and tissues.

EXPERIMENTAL

Materials—Water was distilled from alkaline permanganate in an all-glass distillation apparatus. Cellulose dialysis tubing[1] and crystalline rabbit serum albumin[2] were used.

Adult, male albino rabbits[3], New Zealand strain, were the experimental animals. No special pretreatment methods or diets were incorporated into the *in vivo* procedures.

The following chemicals were used: methylprednisolone[4], sulfisoxazole[5], and *N*-1-naphthylethylenediamine dihydrochloride[6]. All other chemicals were either USP or reagent grade and were used as received.

Equilibrium Dialysis—The cells and procedures used were described previously (1). Protein solutions or eye fluid and tissue samples were placed in one half-cell chamber, with or without the competitive inhibitor, and the drug solution was placed in the other half of the dialysis cell. The dialysis membrane preparation and the drug and dialysis solution preparation were described previously (1).

Assays—Analytical procedures were described previously (1).

Miosis–Time Studies—A complete description of procedures used in the pupillary diameter measurement was given previously (1). The competitive inhibitor was added directly to the drug solution prior to instillation into the rabbit eye.

[1] Union Carbide Corp., Chicago, Ill.
[2] Schwarz/Mann, Orangeburg, N. Y.
[3] Klubertanz, Edgerton, Wis.
[4] Gift of The Upjohn Co., Kalamazoo, Mich.
[5] Gift of Hoffmann-La Roche, Inc., Nutley, N. J.
[6] Eastman Organic Chemicals, Rochester, N. Y.

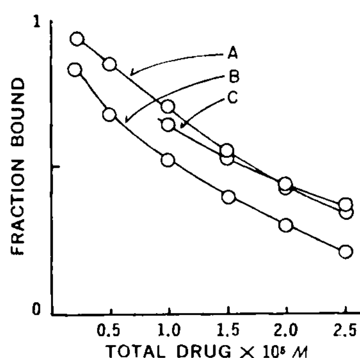

Figure 1—*Equilibrium dialysis experiments on the extent of binding of sulfisoxazole to rabbit serum albumin, alone and in combination with the competitive inhibitors sodium lauryl sulfate and polyoxyethylene (23) lauryl ether. The data points are mean values from four separate determinations, and standard deviations are within ±5% of the mean values. Key: A, drug only; B, drug plus 0.01% sodium lauryl sulfate; and C, drug plus 0.01% polyoxyethylene (23) lauryl ether.*

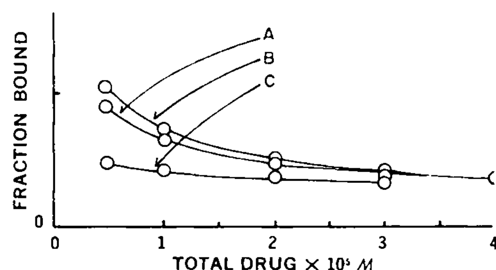

Figure 2—*Equilibrium dialysis experiments on the extent of methylprednisolone binding to rabbit serum albumin, alone and in combination with the competitive inhibitors sodium lauryl sulfate and polyoxyethylene (23) lauryl ether. The data points are mean values from four separate determinations, and standard deviations are within ±5% of the mean values. Key: A, drug only; B, drug plus 0.01% sodium lauryl sulfate; and C, drug plus 0.01% polyoxyethylene (23) lauryl ether.*

RESULTS

***In Vitro* Demonstration of Competitive Inhibition of Drug–Protein Interaction**—A commonly used method to illustrate competitive inhibition of drug-protein interactions is equilibrium dialysis. The increase in free drug concentration in the presence of a competitive inhibitor reflects competitive inhibition. This is illustrated in Fig. 1 for sulfisoxazole and rabbit serum albumin, using sodium lauryl sulfate and polyoxyethylene (23) lauryl ether[7] as competitive inhibitors. Sodium lauryl sulfate is an effective competitive inhibitor in this system, whereas polyoxyethylene (23) lauryl ether is not. Figure 2 shows where polyoxyethylene (23) lauryl ether is a successful inhibitor of the binding of methylprednisolone to rabbit serum albumin. These examples illustrate the structural and charge requirements for successful competitive inhibition of a particular drug-protein interaction. Ideally, the competitive inhibitor should bind to the same site normally occupied by the drug molecule and exhibit a greater affinity for that site than the drug. A further description of structural requirements for competitive inhibitors is available (4) and will be discussed subsequently.

An extensive search for an optimum competitive inhibitor for a particular drug was not conducted in the present study. The study objective was to show successful competitive inhibition in ophthalmic systems. For this purpose, a competitive inhibitor was selected on the general basis that it should have a similar charge type as the drug in question and an affinity for albumin that is substantially larger than that of the drug. With respect to this last point, an inhibitor was chosen that had reasonable aqueous solubility and, at the same time, possessed a hydrophobic chain for stabilization of the drug-protein complex when formed. All of these requirements seem to be met with the surfactant class of compounds. Competitive inhibition of drug binding to rabbit serum albumin was used as a qualitative yardstick to select competitive inhibitors.

Since all competitive inhibitors reported in this study are surface active and can associate to form micelles in aqueous solution, consideration was given to the concentration of surfactant used. Micelle formation would considerably increase the complexity of these systems and make interpretation of the results more difficult (5–7).

The concentration of surfactant used in all *in vivo* experiments was below the reported (8) critical micelle concentration (CMC). However, considering the decrease in CMC that results from addition of salts and buffer components, it is probable that the instilled solutions were above the CMC. Upon instillation of the drug solution, there is a dilution with tears and an elevation in solution temperature to that of the body, which, when coupled with tear turnover, should reduce the micellar influence.

Competitive inhibition of drug-protein binding in ocular tissues and fluids is also possible and can be demonstrated. Figure 3 shows the binding of methylprednisolone to the components of human tears in the presence and absence of polyoxyethylene (23) lauryl ether. The sizable decrease in fraction of methylprednisolone bound to tear protein in the presence of polyoxyethylene (23) lauryl ether is apparent and, when extrapolated to a living system, suggests a considerable increase in bioavailability for this drug. The increase in bioavailability to the eye is particularly significant when it is realized that solution drainage from the eye is very rapid and that the rate of tear turnover is quite large (1, 9).

A significant decrease in fraction bound is also observed for drugs that are not highly bound to proteins (1), such as the miotic drug pilocarpine nitrate. Figure 4 illustrates the fraction of pilocarpine bound to the components of plasmoid aqueous humor in the presence and absence of the competitive inhibitor cetylpyridinium chloride. In the presence of cetylpyridinium chloride, the data points fall on the abscissa axis, showing that complete inhibition of binding occurs. Plasmoid aqueous humor was obtained by paracentesis and has the property of being very rich in proteins as compared to normal aqueous humor. Cetylpyridinium chloride is commonly used as a preservative in drug delivery systems, but its structural features make it an efficient competitive inhibitor for binding of certain cationic drugs to proteins.

***In Vivo* Demonstration of Competitive Inhibition of Drug–Protein Interaction**—Successful competitive inhibition of drug-protein binding in eye fluids and tissues should result in an increase in free drug concentration and an increase in the corneal transport rate of the drug in question. The elevated concentration of free drug should be demonstrable in an *in vivo* system through either an increased biological response to the drug or actual measurement of drug concentrations.

To demonstrate the increased biological response, the miotic drug pilocarpine nitrate was selected and pupillary diameter change was used as the biological response. Figure 5 shows miosis–time profiles for several concentrations of pilocarpine nitrate, with and without cetylpyridinium chloride, in albino rabbits. Several interesting aspects to these curves will be explored in a separate publication,

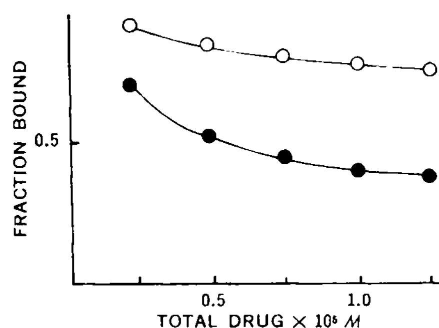

Figure 3—*Equilibrium dialysis experiments on the extent of methylprednisolone binding to the components of human tears in the presence and absence of polyoxyethylene (23) lauryl ether. Key: ○, drug only; and ●, drug plus polyoxyethylene (23) lauryl ether. The data points are mean values from three separate determinations, and standard deviations are within ±7% of the mean values.*

[7] Brij-35, ICI America, Inc.

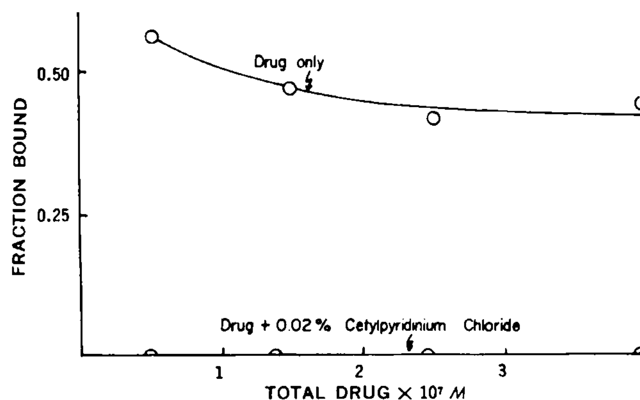

Figure 4—*Equilibrium dialysis studies of tritiated pilocarpine nitrate binding to plasmoid aqueous humor in the presence and absence of 0.02% cetylpyridinium chloride. The data points for the cetylpyridinium chloride study fall on the abscissa axis since there was no binding. Each data point represents the mean values from two determinations and has a standard error equal to or less than 10% of the mean value.*

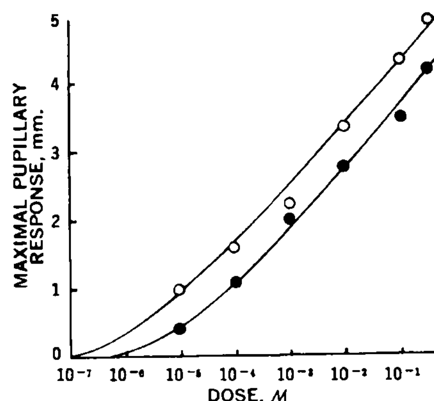

Figure 6—*Maximal miotic response of the rabbit eye to pilocarpine nitrate, with and without 0.02% cetylpyridinium chloride, as a function of dose. Key:* ●, *drug alone; and* ○, *drug plus 0.02% cetylpyridinium chloride.*

but only the relationship between dose and maximum response is of interest here.

Addition of cetylpyridinium chloride to the pilocarpine nitrate solution prior to instillation increases the miotic effect at all concentrations tested. In Fig. 6, the maximum change in pupillary diameter was used as the biological response to generate a dose–response relationship. The log dose–response curve appears to be sigmoidal in shape, with the upper limit being set by either saturation of all existing receptor sites or the smallest pupillary diameter attainable while the lower end is no response at all. An upper limit was not reached due to several factors associated with the pilocarpine nitrate solution, such as approaching the solubility limit of the drug in the buffer at the 5×10^{-1} *M* dosage concentrations and encountering other severe, concomitant drug responses at the higher dosing levels. Nevertheless, the log dose–response relationship is linear over several orders of magnitude.

As can be seen from Fig. 6, the activity of pilocarpine nitrate was increased by a factor of 10 when 0.02% cetylpyridinium chloride was added to the system. Thus, 10 times less drug is required to achieve the same pupillary diameter when the competitive inhibitor is added to the drug solution. Instillation of cetylpyridinium chloride by itself had no effect on pupillary diameter.

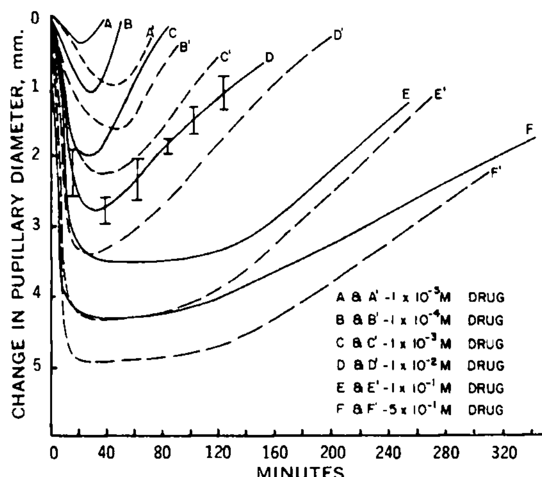

Figure 5—*Miosis–time profiles for various concentrations of pilocarpine nitrate, with and without 0.02% cetylpyridinium chloride. Key: ——, pilocarpine nitrate alone; ---, pilocarpine nitrate plus competitive inhibitor. Standard deviation lines are shown for the* 1×10^{-2} M *drug case only for the sake of clarity. All other lines have similar standard deviations. Each concentration line represents a minimum of four separate determinations in four different animals. Each line was constructed on the basis of 15–40 experimental points.*

Examination of the corneal surface with fluorescein solution and UV light, following repeated application of this concentration of cetylpyridinium chloride for several days, showed no visual damage to the epithelial layer of the cornea.

DISCUSSION

Very small amounts of an instilled drug are transported across the cornea, due in part to the rapid drainage of an instilled solution and to rapid tear turnover (9). Both of these processes cause removal of drug from the precorneal area. If a drug undergoes extensive binding to proteins in tears or to precorneal eye tissue, the amount of free drug available to exert a local effect or to be absorbed across the cornea is reduced significantly. Moreover, the very efficient removal of both free and bound forms of the drug from this area is responsible for a considerable loss in drug and a reduction in drug activity. Similarly, binding of drug to the cornea reduces the transport rate of drug through the cornea, which, when coupled with the turnover of aqueous humor, can greatly reduce the concentration of drug in the anterior chamber of the eye. Therefore, competitive inhibition of this drug–protein binding, especially in the precorneal area, should result in a considerable increase in the bioavailability of drugs that are normally bound to proteins.

The *in vivo* data presented show that addition of certain substrates to ophthalmic drug solutions increases the rate of corneal transport for these drugs as evidenced by miotic increases. The *in vitro* equilibrium dialysis data demonstrate that these substrates are able to serve as competitive inhibitors of drug–protein binding. It is our belief that a portion or all of the absorption rate enhancement through the addition of "biologically inert substances" to the drug solution is through competitive inhibition of drug–protein interaction. The data strongly support the suggestion that enhanced corneal penetration of drugs by addition of certain substrates is through a competitive inhibition mechanism. However, the data do not preclude all other possible mechanistic interpretations.

Because of the structural requirements for competitive inhibition of drug–protein binding, all substrates used were surface active and fall in the surfactant class of compounds. That surfactants can influence the extent of corneal absorption of drugs is well known (10), but this influence has not been attributed to competitive inhibition of drug–protein binding.

Surfactant influence on drug absorption in various parts of the body (11, 12) including the eye (10, 13, 14) is well established. What is not well established is the mechanism for this effect. Such factors as drug solubilization above the CMC, wetting of the membrane surface, and structural modification of the membrane have all been indicated in promoting or retarding drug absorption through biological membranes (5, 6, 15). However, the influence of premicellar concentrations on drug transport has not been studied thoroughly, and any influence on drug transport rate is generally ascribed to changes in the membrane.

It is our belief that competitive inhibition of drug–protein binding is at least partially responsible in many cases of enhanced drug transport. For example, it has been shown that surfactants induce

structural changes in proteins (16) and in biological membranes (13) which, in terms of membrane transport, can be interpreted as an enlargement of either the effective pore size in the membrane or the area of the membrane, thus leading to a rate enhancement. An alternative explanation is that the protein modification leads to a decrease in available binding sites and, hence, less protein binding of the drug.

Consider the possible alternative mechanisms of enhanced corneal transport in more detail. The influence of detergents on proteins varies considerably as a function of pH, detergent structure and concentration, and type of protein. Some detergents such as sodium lauryl sulfate induce large changes in the protein (17, 18), and their competitive inhibition activity in equilibrium dialysis experiments is usually ascribed to this protein denaturation. With sodium lauryl sulfate, this influence exists both above and below the CMC. However, some surfactants do compete for drug binding sites in the classical sense (16). In the present study, several surfactants were used but the principal agent was cetylpyridinium chloride. This compound was used in all experiments at a concentration of 0.02%. Neither the data in this report nor the data in the literature elucidate the mechanism of its competitive inhibitory activity. Thus, whether this agent is merely competing for the binding sites normally occupied by the drug or is removing the binding sites through an unfolding mechanism is unknown. Irrespective of the mechanism, the net result of the interaction, namely, competitive inhibition of drug-protein binding, is the important point. The remaining question is whether competitive inhibition in tears is responsible for the entire effect or is it necessary to include alteration of the epithelial layer of the cornea. If inclusion of corneal tissue changes is necessary, what is the mechanism of transport *via* this change? Is it competitive inhibition *via* protein denaturation or is it a physical enhancement as described earlier?

The *in vivo* activity of pilocarpine nitrate plus 0.02% cetylpyridinium chloride can be significantly decreased by addition of albumin to the drug–cetylpyridinium chloride solution, suggesting that drug-protein binding can alter the availability of free drug (1). However, raising the concentration of cetylpyridinium chloride in the drug–albumin solution restores the biological activity so that a fully reversible system can be established and the reversibility is controlled either by albumin or cetylpyridinium chloride. This supports competitive inhibition in the tear film as a contributing factor in the enhancement of drug activity.

Further support for competitive inhibition of drug binding as a principal mechanism was obtained using the anionic steroid fluorometholone. Addition of cetylpyridinium chloride to an albumin-fluorometholone solution in an equilibrium dialysis experiment does not raise the free drug concentration, as expected, since the steroid is of neutral charge as compared to cetylpyridinium chloride and would therefore occupy different sites. In a similar vein, cetylpyridinium chloride does not promote corneal absorption of this drug[8]. If changes in the corneal tissue by addition of cetylpyridinium chloride were responsible for promoting or inhibiting drug penetration, it is expected that all drugs would be affected, albeit to different extents. It is, of course, possible that the different drugs penetrate the cornea *via* different routes, *i.e.*, intercellular *versus* intracellular, and the membrane changes caused by cetylpyridinium chloride do not influence the pathway through which fluorometholone is absorbed.

Data supporting a corneal membrane effect for cetylpyridinium chloride and other agents are given by Green and Tonjum (13). Using *in vitro* corneal transport studies, they showed that application of surfactants such as cetylpyridinium chloride and benzalkonium chloride to the cornea, prior to application of a fluorescein solution, results in an increased penetration of fluorescein. Based on electron micrographs, the mechanism of enhanced penetration was ascribed to an increased intercellular space area in the outermost epithelial layer of the cornea, analogous with the effects of ouabain as well as high endothelial pressure, both of which lead to an increased rate of drug penetration.

There are major differences in experimental technique between the study of Green and Tonjum (13) and the present work, and it is important to consider these differences in trying to assign a mechanism of action. Green and Tonjum used concentrations of cetylpyridinium chloride that were well above the CMC (8). A conclusion as to whether or not they were above the CMC with benzalkonium chloride cannot be reached without knowing the specific structure of the quaternary ammonium compound used. In addition to the differences in initial cetylpyridinium chloride concentration, there was a difference in contact time and concentration during this contact time. Green and Tonjum soaked the excised cornea in the surfactant solution for 2 min. prior to application of the fluorescein solution. This approach yields a significantly higher effective surfactant concentration than do experiments on an intact animal, where tear and instilled solution drainage as well as tear turnover significantly alter the surfactant concentration (9). Thus, concentrations of cetylpyridinium chloride above the CMC as well as the higher effective concentration for longer periods might as well lead to membrane changes that do not occur at lower concentrations in *in vivo* experiments.

Due to the differences in the experimental techniques of Green and Tonjum and the present study, a definite conclusion as to mechanisms apparently cannot be made. It is, of course, possible to interpret the results of Green and Tonjum in terms of competitive inhibition; *i.e.*, the increased intercellular area is merely a form of protein unfolding and therefore binding sites are masked as opposed to the more physical interpretation of a greater area for drug penetration.

In summary, it would appear that competitive inhibition of drug–protein binding is at least partly responsible for an increase in drug transport rate when some surfactants or competitive inhibitors of drug-protein binding are incorporated into the drug delivery system. It is probable, based on the work by Green and Tonjum (13), that cetylpyridinium chloride and similar substrates under suitable conditions owe part of their drug transport potentiation property to changes in the epithelial layer of the cornea, although the specific mechanism is unknown.

All work described in this report was carried out on healthy eyes. Diseased eyes will show a significant increase in precorneal protein binding and are better candidates for competitive inhibition of drug-protein binding.

[8] Unpublished data from this laboratory.

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