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Studies on Corneal Penetration of *P*-Hydroxybenzoic Acid Esters

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Abstract □ Corneal permeability of various *n*-alkyl *p*-hydroxybenzoates (parabens) was studied *in vitro* using excised rabbit corneas, and the effect of lipophilicity of parabens on the corneal permeability was also investigated. Permeability coefficients were obtained from the least-square linear regression after the steady state had been reached. Lipophilicity of parabens was calculated by distribution coefficients determined in octanol-S | 12φ‖) rensen's buffer solution (pH 5.0). The relationship between lipophilicity and corneal permeability of parabens was not linear, but the optimum lipophilicity for the maximum permeation was found. The influence of tween 80 on corneal permeability of methyl and butylparaben was not significant.

Keywords □ Corneal permeability, excised rabbit corneas, *n*-alkyl *p*-hydroxybenzoates, distribution coefficients, lipophilicity, permeability coefficients, tween 80.

Whenever an ophthalmic drug is applied topically to the eye, only a small amount actually penetrates the cornea and reaches the internal tissues. Typically less than 3% of an applied dose penetrates to the aqueous humor[1]. Therefore, a major problem in ocular therapeutics is the poor bioavailability of an applied durg. This results from a short residence time of drugs in the eye, a high impermeability through the cornea, and a non-productive absorption into the conjunctiva and sclera[2].

Drug absorption across cornea is determined by the physicochemical properties of an applied drug and the cornea itself. The cornea is composed of three distinct major layers, each covering the entire convex corneal area; from anterior to posterior, these are the epithelium, the stroma and the endothelium. Epithelium and endothelium, being lipoidal in nature, may represent at diffusional barrier offering high resistance to ionic or relatively hydrophilic species. And the stroma, being hydrophilic in nature, is made up of collagen fabrics, which are arranged parallel to the corneal surfaces in such manner that the cornea is transparent at normal thickness[3].

Therefore, depending on the physicochemical properties of an applied drug, the diffusional resistance offered by the individual layers can vary greatly. This has been shown for the rabbit cornea by Schoenwald[4], Huang[5,6], Hansch[7], and Chrai *et al.*[8-18]. Among the physicochemical properties of drug, lipophilicity is an important molecular property influencing the pharmacokinetic and pharmacodynamic behaviour of many classes of drugs[19-24]. Moreover, among the different lipophilicity descriptors, partition coefficients are particularly significant.

However, little is known about the relationships between lipophilicity and transport rate of drugs across cornea. The objective of this study is to determine if the corneal permeability coefficients of parabens can be correlated with their lipophilic characteristics. A homologous series of parabens with different length of the alkyl group of *p*-hydroxybenzoic acid esters were used in this investigation using albino rabbit as an animal model.

EXPERIMENTAL SECTION

Materials

p-Hydroxybenzoic acid (HBA) and *n*-alkyl *p*-hydroxybenzoates; methylparaben (MHB), ethylpara-

ben (EHB), propylparaben (PHB), and butylparaben (BHB) were purchased from Sigma Chem. Co., and *n*-amylparaben (AHB) and *n*-hexylparaben (HHB) from Tokyo Kasei, and water was purified with a Milli-Q water system (Millipore, Bedford, MA., USA). Methanol, acetonitrile, and octanol obtained from Merck Sharp & Dohme Res. Lab. (West Point, PA.) were HPLC grade. All other reagents were of analytical grade. Isotonic Sφrensen's buffer solution (pH 5.0) was prepared from monobasic potassium phophate and dibasic sodium phosphate, by adding sodium chloride. Male New Zealand white rabbits, weighing between 2.0 and 2.5 Kg, were used for the experiments.

Apparatus

A schematic diagram of the methacrylate apparatus with two chambers is given in Fig. 1. The effective surface area for drug permeation is 0.636 cm^2. Excised cornea was mounted between two chambers, and the tear and aqueous humor sides of the chamber were filled with 3 m*l* of sample solution and iostonic Sφorensen's buffer solution, respectively.

Solubility of parabens

An excess amount of HBA and parabens were allowed to eqilibrate with Sφrensen's phosphate buffer solution (pH 5) in a sealed vial with gently shaking for 5 days at 35℃. After equilibration, the samples were filtered through a 0.22 μm microporous filter (Millipore, Bedford, MA., USA) which was pre-warmed at 35℃. Analysis of the filtrate was performed at 254 nm using UV spectrophotometer (L.K. B., Model 4050, U.K.).

Partition coefficients

Sφrensen's phosphate buffer solution (pH 5.0) and octanol were mutually saturated at 35℃ before use. The distribution coefficients for HAB, MHB, EHB, PHB, and BHB were determined by dissolving paraben 1000 μg in 10 m*l* of pH 5 phosphate buffer and shaking intermittently with 2 m*l* of octanol kept at 35℃ for 3-10 days to reach a distribution equilibrium. For AHB and HHB, 500 μ*l* of octanol was used in determining distribution coefficients. The volume ratio of octanol and Sφrensen's buffer was depended on the lipophilicity of HAB and various parabens. The phases were separated by centrifugation at 2500 rpm for 10 min, and the concentration in each phase was determined spectrophotometrically. The volumes of each phase were chosen so that the concentration of HAB and parabens in the aqueous phase, before and after extraction, could be measured by spectrophotometer. The distribution coefficient was calculated by Eq. 1[4]:

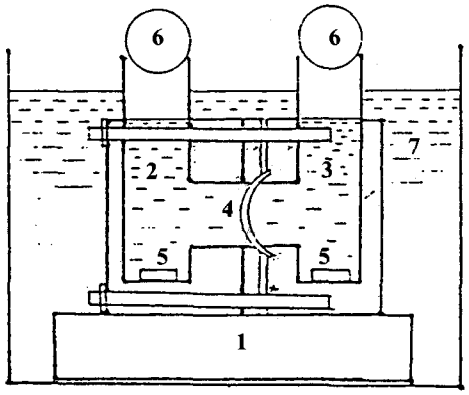

Fig. 1. Schematic diagram of methacrylate diffusion cell for corneal permeability.
Key 1; stirrer, 2; tear side, 3; aqueous humor side, 4; cornea, 5; magnetic bar, 6; glass ball, and 7; water bath (35℃).

$$\text{Distribution coefficient (DC)} = \frac{[Cb-Ca]\,V_W}{[Cb]\,V_o} \quad (1)$$

where Cb and Ca represent the concentration of parabens in Sφrensen's buffer phase before and after distribution, respectively; V_o and V_W represent the volume of octanol and Sφrensen's buffer phases, respectively.

The partition coefficient was calculated from the distribution coefficient by Eq. 2;

$$\text{Partition coefficient (PC)} = DC\left[1+\frac{1}{\text{antilog}\ (pH-pKa)}\right] \quad (2)$$

All distribution coefficients reported here were measured at pH 5.0. The pH of the buffered phase at 35℃ was not changed after distribution was complete. The pKa of parabens was 8.45 and that of HBA was 4.36.

Excised cornea procedure

Male New Zealand white rabbit was sacrified by

injecting a bolus of air into the marginal ear vein. The intact eye, along with the lids and conjunctival sac, was then enucleated. The sclera was cut around the cornea; then the cornea was separated from the lens, iris, and vitreous body. The endothelial surface of the cornea was gently rinsed with saline and extreme care was taken not to produce any wrinkles or folding of the cornea. And the exposed cornea was carefully mounted between two chambers of diffusion apparatus within 20 min from the time of sacrificing the animal.

Determination of corneal permeability

After mounting the transparent cornea between two chambers of diffusion apparatus, the tear and aqueous humor sides of the chamber were filled with 3 m*l* of sample solution and isotonic Sφrensen's buffer solution (pH 5), respectively. The sample solutions containing 100 μg of parabens/m*l* were made by dissolving parabens in Sφrensen's buffer and the solutions were pre-equilibrated to the temperature of the study (35℃). The chamber was incubated in a water bath at 35℃, which is the living temperature of rabbit cornea, and was suitably stirred with magnetic stirrers. A mixture of O_2: CO_2 (95 : 5) was bubbled through the drug free solution 10 min prior to use.

A sample solution volume of 1 m*l* was withdrawn through the sample port at the top of the receptor chamber periodically over a 4-hr period, starting at 10 min following the beginning of an experiment. An equal volume of Sφrensen's beffer was immediately added to the receptor chamber to maintain a constant volume.

The influence of a surfactant (tween 80) on the corneal permeation of parabens was also studied according to the same method as described above. The concentraion of tween 80 used was 0.01 v/v% in isotonic Sφrensen's buffer solution.

Corneal thickness

After the permeation experiment, the cornea was taken from the diffusion apparatus and the wet weight of cornea was measured. Then, the cornea was dried in an oven at 103℃ for 24 hr and the dry weight was measured so that the hydration level of the cornea during steady state could be determined. From these values, the corneal hydration was calculated using Eq. 3:

$$\text{Hydration level (HL)} = \frac{\text{Wet weight} - \text{Dry weight}}{\text{Dry weight}} \quad (3)$$

And, for a 2-kg rabbit, the thickness of the cornea can be determined by Eq. 4[5]:

$$\text{Thickness of cornea (TH)} = \frac{0.42 + (\text{HL})}{100} \quad (4)$$

where (HL) represents hydration level. The rabbit cornea can be divided into three distinct diffusional layers. The outer (epithelium), consisting of 6-10 cellular layers, is the most lipophilic. The inner layer, which is also lipophilic, consists of a single layer of endothelial cells. The middle layer (stroma) is a hydrophilic layer which accounts for 90% of the corneal thickness. The epithelium and endothelium control hydration and therefore normal thickness; however, when the cornea swells it is the stromal layer only which collects fluid and swells. Consequently, for a 2-kg rabbit, the epithelial and endothelial thickness remain constant at 0.00385 and 0.0005 cm, respectively[5]. From these values and the experimentally determined hydration levels, the coreal thickness was computed by Eq. 4.

Calculation of permeability coefficients

The apparent permeability coefficient (Papp, cm/sec) was determined by Eq. 5[25]:

$$\text{Papp} = \frac{\Delta Q}{\Delta T \; A \; 60 \; Co} \quad (5)$$

where the term $\Delta Q/\Delta T$ is the permeability rate (i.e., steady-state flux, μg/min) of parabens across each excised cornea, Co is the initial paraben concentration (μg/m*l*), A is the corneal surface area (cm^2), and (60) is the constant to convert minute to second.

Drug assay

An HPLC method was used for analysis of parabens. The HPLC system (Waters Associates, Milford, MA 01757) equipped with a data module (Model 730), a solvent delivery system (Model 510), a variable wavelength UV detector (Model 481), and an automated gradient controller was used as the following conditions; column, Lichrospher 100 RP-18 (5 μm) (Cat. No. 50943, Merch Sharp & Dohme Res. Lab., Rahway, NJ.); mobile phase, Sφrensen's

Table I. Solubility of HBA and parabens in Sørensen's phosphate buffer and the Octanol-Sørensen's buffer partition coefficients (PC) at 35°C.

	HBA	MHB	EHB	PHB	BHB	AHB	HHB
Solubility (mg/ml)	10± 0.14	2.98± 0.07	1.25± 0.02	0.48± 0.01	0.30± 0.01	0.26± 0.02	0.05± 0.003
Log PC	0.89	5.31	5.60	6.03	6.29	6.73	7.25

*Each value represents the mean of three measurements (± SD)

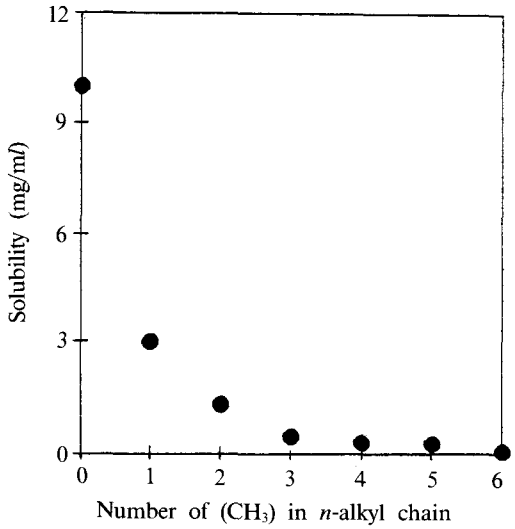

Fig. 2. Relationship between the solubility and the length of *n*-alkyl chain, $(CH_3)n$, of HBA and various parabens at 35°C.

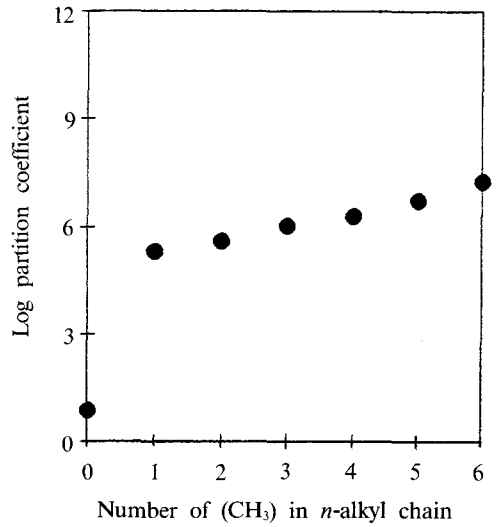

Fig. 3. Relationship between the octhanol-Sørensen's buffer partition coefficients and the length of *n*-alkyl chain, $(CH_3)n$, of HBA and various parabens at 35°C.

phosphate buffer (pH 5): Methanol (7 : 3) for HB, Sørensen's phosphate buffer (pH 5): methanol (1 : 1) for MHB and EHB, Sørensen's phosphate buffer (pH 5): acetonitrile (1 : 1) for PHB and BHB, and Sørensen's phosphate buffer (pH 5): acetonitrile (4 : 6) for AHB and HHB; flow rate, 0.9 ml/min; sensitivity, 0.005 AUFS; injected volume, 25 μl; detector, 254 nm. Peak height was employed for the determination of drug concentrations.

RESULTS AND DISCUSSION

Solubility and partition coefficients

The solubilities of parabens in Sørensen's phosphate buffer solution (pH 5) at 35℃ are listed in Table I. The solubility of parabens decreases with an increase in the length of *n*-alkyl chain (methyl groups), from HBA to HHB. And the relationship between the solubility in Sørensen's buffer and the length of *n*-alkyl chain of parabens is illustrated in Fig. 2. From these results, it is clear that the aqueous solubility of parabens decreases with an increase of the lipophilicity as resulted from the addition of methyl groups.

Lipophilicity is a molecular property, which can be factorized into two terms, one representing bulk or steric properties, while the other is related to electrostatic and polar properties[26]. Therefore, lipophilicity used in this report is expressed as a partition coefficient among the different lipophilicity descriptors. The partition coefficients of parabens were calculated by Eq. 2 from the distribution coefficients determined in the octanol-Sørensen's phosphate buffer system at 35℃. Table I also lists partition coefficients of parabens and shows that the lipophilicity of parabens increases with an increases in the length of *n*-alkyl chain, as shown in Fig. 3.

Table II. Permeability coefficients and hydration levels for the permeation of HBA and parabens, across excised cornea

	HBA	MHB	EHB	PHB	BHB	AHB	HHB
$\Delta Q/\Delta T$ (ug/ml · min)	0.008	0.041	0.037	0.040	0.030	0.009	0.001
	± 0.002	± 0.002	± 0.004	± 0.005	± 0.003	± 0.005	± 0.0004
Papp (cm/sec × 10^{-5})	0.21	1.07	0.97	1.05	0.79	0.47	0.10
	± 0.005	± 0.05	± 0.09	± 0.13	± 0.08	± 0.26	± 0.04
Hydration	4.76	4.49	4.26	4.33	4.59	4.74	4.77
levels	± 0.65	± 0.38	± 0.64	± 0.28	± 0.52	± 0.92	± 0.19

*Each value represents the mean of five measurements (± SD)

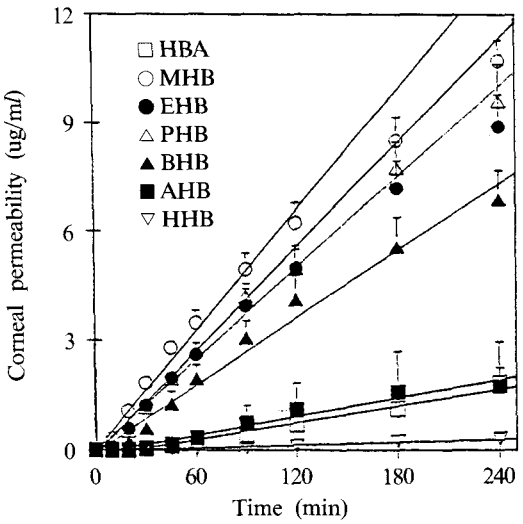

Fig. 4. The permeation profiles of HBA and various parabens across excised rabbit cornea.

Corneal permeability

Fig. 4 represents the permeation profiles of HBA and parabens across excised rabbit cornea. The data points closely fit the least-square regression line ($r > 0.96$) after steady state has been reached. The permeation profile of parabens shows a short lag time, as illustrated in Fig. 4. The lag time, defined by the linear intercept on time axis, is related to the time required to reach steady-state permeation. Generally, the more rapidly penetrating drugs will have a sho- the more rapidly penetrating drugs will have a shorter lag time and a greater state flux.

The slope of the straight line ($\Delta Q/\Delta T$), which is the permeability rate or the rate of parabens quantity penetrating the cornea at time t, was substituted into Eq. 5 to obtain an apparent permeability coefficients (Papp). The Papp of parabens obtained from these experiments are given in Table II. From Table II and Fig. 4, it is known that the permeability rate of MHB is 0.041 μg/min and it is greater than the others.

Table II also lists the hydration levels obtained from each excised rabbit cornea. Excised intact cornea maintained its transparency and did not exceed a hydration level of 50% after 4 hr of permeation. And it was less hydrated in Sørensen's buffer solution (pH 5) than 0.9% sodium chloride solution. The corneal thickness calculated by Eq. 4 from the average of these hydration levels was approximately 0.049 cm.

Permeability versus lipophilicity correlations

The permeation of drugs across the cornea may achieve through two different pathways that depend on the physico-chemical characteristics of the applied drug in the eye[11,27]. First is the paracellular pathway through intercellular spaces of epithelium and endothelium. This pathway appears similar to drug movement in an aqueous environment. Compounds having very low molecular weight and small ions demonstrate rapid uptake into the aqueous humor despite the lipid-like barrier imposed by the corneal epithelium. The limiting size of the paracellular pathway appears to be in the order of 60 Å or less, the molecular size of glycerol. Second, intercellular spaces is quite small, so most drugs would go through the transceular pathway in crossing the cornea. The basic factors governing drug permeation *via* transcellular pathway are the lipophilicity of drug, the pKa which controls the proportion of drug absorbed at given pH, and molecular size. The physico-chemical characteristics of a drug that probably have the greatest effect on corneal permeation through the transcellular pathway

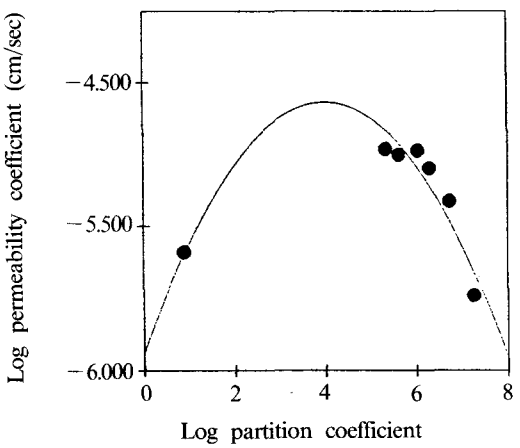

Fig. 5. Log-log plot of permeability coefficient versus partition coefficient (Octanol-Sφrensen's buffer, pH 5.0).

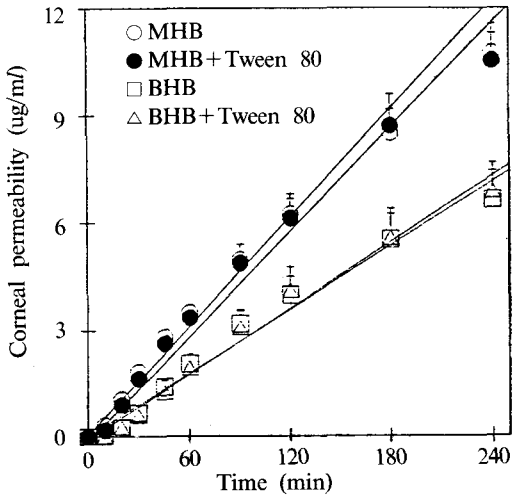

Fig. 6. The permeation profiles of MHB and BHB with and without 0.01 v/v% Tween 80.

Table III. Permeability coefficients and physical constants of HBA and parabens

	log Papp (cm/sec)	Log (DC)	Log (PC)	Log (MW)
HBA	−5.68	0.80	0.89	2.14
MHB	−4.97	1.86	5.31	2.18
EHB	−5.01	2.15	5.60	2.22
PHB	−4.98	2.69	6.03	2.26
BHB	−5.10	2.95	6.29	2.29
AHB	−5.33	3.28	6.73	2.32
HHB	−5.98	3.81	7.25	2.35

are the relative lipid and aqueous affinities.

In the permeation of parabens across the cornea, it dose not seem to be the paracellular pathway, because parabens have greater molecular weights (MW. 138-194) than that of glycerol (MW. 82), a limiting size of paracellular pathway. Since parabens, except HBA (pKa 4.35), being its pKa 8.45, also do not ionize in Sφrensen's buffer (pH 5), it can be recognized that the permeation processes of parabens will be governed by its lipophilicity. Fig. 5 shows a plot of log (Papp) against log (PC), and Table III contains the calculated parameter values. The lipophilicity of parabens increases with an increase in the length of *n*-alkyl chain as resulted from the addition of methyl groups, but the permeability is not proportional to its lipophilicity.

In Fig. 5 as discussed by Schoenward[28] and Mosher[29], the increase of the permeability does not go on indefinitely with an increase in the lipophilicity of the paraben series. The permeability coefficient of HHB having partition coefficient of 7.25 is lower than that of HBA with the smallest partition coefficient of 0.89. Considering the cornea composed of three distinct layers with the different lipophilicity, it is clear that the increase of lipophilicity is important to improve drug permeation across the cornea as shown in Fig. 5. But it is known that there is a plateau region representing the change of rate-limiting layer. Several reasons could be given for these experimental results. Strictly speaking, for the very lipophilic drugs, the permeability is probably controlled by the hydrophilic stroma and for the more hydrophilic drugs, the epithelium acts as a significant barrier to drug permeation. Therefore, it is suggested that since a drug must penetrate corneal layers having different polarity and structural characteristics, it should have some degree of solubility in both of membrane layers.

Mutiple regression analyses[4] were performed on the data to find the best set of parameter to describe the change of log (Papp) to a change of log (PC). And a $(\log PC)^2$ term was also included, because of the plateau region as shown in Fig. 5. The regression analysis of the best fit for log (Papp) was represented in Eq. 6:

$$\log (Papp) = -6.379 + 0.872 \log (PC) - 0.109 [\log (PC)]^2, \quad r = 0.97 \qquad (6)$$

Eq. 6 predicts an optimum permeability, log (PC) value of 4.0. However, there is no experimental evidence that a parabola would best describe the data, because a compound having log (PC) value of 4.0 could not be obtained to test this phenomenon.

The influence of tween 80 (0.01 v/v%) on corneal permeability of MHB and BHB was also investigated, but not significant. Fig. 6 shows the effect of tween 80 on permeability rate of MHB and BHB.

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