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Research Article

Determination of the pH-Dependent Phase Distribution of Prostaglandin E_1 in a Lipid Emulsion by Ultrafiltration

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The distribution of prostaglandin E_1 (PGE_1) in a lipid emulsion has been shown to be consistent with a three-phase model which assumes that solute may reside in the bulk aqueous and oil phases and at the oil/water interface. Calculations suggest that, in a lipid emulsion having an average particle size of 0.11 μm, it is theoretically possible for a surface active species such as PGE_1 to exist predominantly at the interface. Aqueous phase concentrations of PGE_1 versus pH were measured in an emulsion having an oil/water phase volume ratio of 0.1 by the use of an ultrafiltration technique in order to estimate the relative percentages of PGE_1 in each phase. From bulk oil/water partition coefficient determinations, the amount of PGE_1 present in the bulk oil phase of the emulsion was concluded to be insignificant. At emulsion pH values less than 5, PGE_1 resides preferentially (>97%) at the interface. With increasing pH's, the percentage of PGE_1 in the aqueous phase increases, reaching 51% at high pH's. A model which assumes that both the nonionized and the ionized PGE_1 species may be present at the interface, depending on the pH, was shown to be consistent with the data. Estimates were made of the distribution coefficients of the ionized and nonionized PGE_1 between the interface and the aqueous phase and their concentration dependence. The apparent pK_a of PGE_1 at the interface derived from these data was 6.8. The distribution coefficients were used to generate a distribution profile of the various PGE_1 species as a function of the pH. This distribution profile will be useful in explaining kinetic data of PGE_1 in the emulsion as a function of pH.

KEY WORDS: lipid emulsion; prostaglandin E_1; phase distribution; ultrafiltration; partition coefficients.

INTRODUCTION

Oil-in-water emulsion formulations of prostaglandin E_1 (PGE_1) have shown both a marked increase in activity in the treatment of various vascular disorders and a reduction in side effects such as local irritation near the site of injection upon parenteral administration (1–5). The emulsion formulation appears to target the drug to the reticuloendothelial system and inflamed tissues. Therefore, commercially marketable emulsion formulations of PGE_1 are of considerable interest.

Since PGE_1 is very labile in aqueous systems and the oil-in-water (o/w) emulsion is approximately 90% water, adequate chemical stability must be demonstrated before this approach can be considered viable. Repta (6,7) has previously shown that agents that are oil soluble and unstable in aqueous media may be stabilized in o/w emulsions. In those studies the overall rate constant for degradation of drug in the emulsion was assumed to reflect the fractional amounts of the drug in the aqueous and oil phases and the rate constants for decomposition in the two phases. This two-phase model may be suitable for many drugs in emulsion systems, but for a surface active molecule such as PGE_1, a three-phase model which accounts for drug residing in the aqueous phase, in the oil phase, and at the oil/water interface may be more appropriate. An important prerequisite in understanding the stability of PGE_1 in emulsion systems, therefore, is the determination of the location of the nonionized and ionized PGE_1 species in the formulation.

In this study, partitioning and ultrafiltration experiments have been employed to determine the relative amounts of PGE_1 in the three phases as a function of the pH. By combining these data with kinetic data, accurate predictions of chemical stability in emulsion systems varying in drug concentration, pH, and phase volume ratio should be possible.

MATERIALS AND METHODS

Materials

All compounds were used as received from the supplier: prostaglandin E_1 (The Upjohn Company), soybean oil (Croda Inc.), soybean phosphatide NC 95 (American Lecithin Co.), glycerin USP (Proctor and Gamble), sodium phosphate monobasic, (J. T. Baker), L-aspartic acid (Nutritional Biochemicals), and absolute ethanol (U.S. Industrial Chemical Co.). All other chemicals were reagent grade.

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PGE_1 Solutions

PGE_1 was dissolved in absolute ethanol to make a 100 mg/ml solution. Ten and fifty microliters of this solution was added to 200 ml of 0.005 *M* aspartic acid buffer at pH 3 to make approximately 5 and 25 μg/ml aqueous solutions of PGE_1. Ten and one hundred seventy microliters of the 100 mg/ml PGE_1 solution was added to 200 ml of 0.005 *M* phosphate buffer at pH 7 to make approximately 5 and 85 μg/ml aqueous solutions of PGE_1.

PGE_1 in the Lipid Emulsion

The lipid emulsion was prepared by adding 1000 ml of water for injection to 21.6 g of soy phosphatide NC 95 (soybean lecithin). This mixture was allowed to stir until the soybean lecithin was completely wetted (approximately 1 hr). One hundred sixteen grams of soybean oil and 26.4 g of glycerin USP were added to the lecithin/water mixture. This total mixture was preemulsified using a Polytron homogenizer (Model PT45/8, Brinkman Industries, Westbury, N.Y.) for 5 min at 1000 rpm. Final emulsification was completed by passing the mixture through a microfluidizer (Model 110, Microfluidics Corp., Newton, Mass.). The equipment settings for the microfluidizer were as follows: air pressure, 80 psi; fluid pressure, 11,000 psi; and number of passes, 15. The emulsion was kept cool during the emulsification process by using a water bath set to approximately 15°C.

The 200 μg/ml concentration of PGE_1 in the emulsion was prepared by adding 0.4 ml of a 100 mg/ml PGE_1 solution in ethanol to 200 ml of emulsion placebo. The mixture was stirred for 15 min to assure uniformity. The pH was adjusted by the addition of either dilute hydrochloric acid or sodium hydroxide. Emulsions at other PGE_1 concentrations were prepared by adding 0.4 ml of the appropriate concentration of PGE_1 in ethanol to 200 ml of emulsion placebo.

Ultrafiltration Methodology

YM series, 62-mm Amicon ultrafiltration membranes were soaked in deionized water, with several changes of water, for at least 1 hr to remove water-soluble extractables. The membranes were placed into a stirred filtration cell (Model 8200, Amicon, Danvers, Mass.) which was jacketed to 35°C with tubing containing 35°C circulating water. The solution or emulsion to be filtered was equilibrated in a stoppered flask at 35°C for 1 hr prior to filtration. After equilibration, 180 ml of the liquid to be filtered was placed into the stirred cell and 20–40 psi of nitrogen was applied to begin the filtration. Approximately 2-ml samples of the filtrate were collected until 15–20% of the liquid had been ultrafiltered. Each sample was assayed for PGE_1 by high-performance liquid chromatography (HPLC). The equilibrated stock solution prior to filtration was also assayed for PGE_1 by HPLC.

HPLC Assay for PGE_1

PGE_1 was determined by an HPLC assay procedure. The HPLC parameters were as follows: column, Brownlee Spheri-5 RP-18; mobile phase, CH_3CN:aqueous 0.002 *M* phosphate buffer (pH 3), 37%:63%; flow rate, 1.0 ml/min; detection wavelength, 200 nm; and standard preparation, 5 to 100 μg/ml in the mobile phase.

Sample Preparation

Aqueous Samples. These samples were injected "as is."

Oil Samples. Approximately 10 g of accurately weighed oil samples was mixed with 10 ml of 95:5 methylene chloride:acetone. The mixture was eluted through a Waters silica gel Sep-Pak. One hundred milliliters of a 95:5 methylene chloride:acetone solution was passed through the Sep-Pak at a rate of about 20 ml/min to remove the oil completely. Twenty milliliters of methanol was used to elute the PGE_1. The methanol elution was evaporated to dryness under nitrogen. Samples were reconstituted with mobile phase prior to HPLC assay.

Oil/Water Partition Coefficient Determination

Five milligrams of PGE_1 was dissolved in 100 ml of soybean oil and allowed to stir for 24 hr. The solution was filtered through a Millex-FG 0.2-μm filter and assayed for PGE_1 using the assay procedure described for oil samples. Approximately 8.5 g (10 ml) of the oil solution was added to 100 ml of water for injection adjusted to pH 3.0 and the oil/water mixture was shaken for 24 hr. The mixture was centrifuged at 3500 rpm for 30 min to separate the layers. Samples of each layer were removed and assayed for PGE_1 by one of the methods listed above.

Particle Size Measurement

The particle size of the emulsion was measured with a photon correlation particle size analyzer (Nicomp Model 200, Pacific Scientific, Silver Spring, Md.).

RESULTS AND DISCUSSION

The two-phase model employed previously (6,7) to describe the kinetics of drug degradation in an emulsion assumes that the drug resides in either the bulk oil phase or the aqueous phase, with the relative amounts in each phase depending on the pH, the pK_a, the phase volume ratio, and the partition coefficient. However, in the case of PGE_1 in a fat emulsion, the two-phase kinetic model may not be appropriate. The partition coefficient of PGE_1 into soybean oil was found in this study to be 0.7. In an emulsion consisting of 10% oil phase, the amount of prostaglandin residing in the bulk oil phase would therefore be a very small fraction of the total and would not account for the improved activity and decreased local irritation of the emulsion upon intravenous administration. An alternative three-phase model which assumes that drug may reside in the aqueous phase, in the oil phase, or at the oil/water interface may therefore be more appropriate.

The small particle size [approximately 0.11 ± 0.04 (SD) μm] of the emulsified oil droplets in the fat emulsions under investigation results in a sizable interfacial surface area. Since PGE_1 is known to possess surface active characteristics (8), one would expect PGE_1 to concentrate at the interface. Providing that sufficient interfacial area is present to

accommodate the PGE_1 present in the emulsion, interfacial PGE_1 may be the predominant term in the mass balance.

Shown below are calculations demonstrating that sufficient interfacial area is available to accommodate the PGE_1 present in the emulsions of interest.

Assumptions for 100 ml (99.55 g) of PGE_1 Fat Emulsion Formulation

1. Formulation is 10% (w/w) (10.8%, v/v) oil [i.e., 10.0 g (10.8 cm^3) of oil].
2. PGE_1 concentration is 5–200 μg/ml.
3. Oil droplet diameter (determined with a Nicomp particle size analyzer) is approximately 0.11 ± 0.04 μm.

Surface area of each droplet
$$= S_d = 4\pi r^2 = 3.8 \times 10^{-10}\ (cm^2)$$

Volume of each droplet
$$= V_d = (4/3)\pi r^3 = 7.0 \times 10^{-16}\ (cm^3)$$

Number of 0.11-μm droplets = N_d
$$= V_{total}/V_d = 10.8\ (cm^3)/7.0 \times 10^{-16}\ (cm^3)$$
$$= 1.5 \times 10^{16}\ \text{droplets}$$

Total surface area = S_t
$$= (N_d)(S_d) = (1.5 \times 10^{16})(3.8 \times 10^{-10})$$
$$= 5.7 \times 10^6\ (cm^2)$$

From surface tension data and the application of Gibb's equation, Johnson and Saunders (8) have estimated the area occupied per prostaglandin molecule at the water surface to be 73 $Å^2$ (= 7.3×10^{-15} cm^2/molecule).

Therefore, the total moles that could go to the interface is

$$(5.7 \times 10^6)/(7.3 \times 10^{-15})(6.02 \times 10^{23})$$
$$= 1.3 \times 10^{-3}\ \text{mol} = 461\ \text{mg}\ PGE_1$$

In a 100-ml emulsion containing 5–200 μg/ml PGE_1 the maximum amount of PGE_1 present would be 20 mg—well below the theoretical amount which could reside at the surface.

It is difficult to measure directly the amount of PGE_1 residing at the interface. However, indirect determination of the PGE_1 at the interface can be made by measurement of the total amount of PGE_1 present in the emulsion and the quantity of unbound prostaglandin in the aqueous phase. Assuming that the partition coefficient of PGE_1 into the emulsion droplet interior is that which was measured experimentally for partitioning into bulk soybean oil, the amount of drug at the interface can be estimated. Shown below is a derivation of the necessary mathematical relationship.

Given that three phases are present, the following terms can be defined.

PGE_1^T = total PGE_1
PGE_1^o = amount of PGE_1 in the oil phase
PGE_1^w = amount of PGE_1 in the aqueous phase
PGE_1^I = amount of PGE_1 at the interface
V_o = volume of the oil phase
V_w = volume of the aqueous phase
E_o = concentration of the PGE_1 in the oil phase
E_w^T = concentration of the PGE_1 in the aqueous phase
E_w^o = concentration of the nonionized PGE_1 in the aqueous phase
E_w^- = concentration of the ionized PGE_1 in the aqueous phase
K_d = partition coefficient (oil/water)

Then, by definition,

$$PGE_1^T = PGE_1^o + PGE_1^w + PGE_1^I$$
$$PGE_1^T = V_oE_o + V_wE_w^T + PGE_1^I$$
$$PGE_1^I = PGE_1^T - [V_oE_o + V_wE_w^T]$$
$$E_w^T = E_w^o + E_w^-$$
$$10^{(pH-pKa)} = E_w^-/E_w^o$$
$$K_d = E_o/E_w^o\ (o/w)$$

Therefore, by appropriate substitution the following equation can be derived:

$$PGE_1^I = PGE_1^T - \frac{(E_w^T)\{V_w[1 + 10^{(pH-pKa)}] + K_dV_o\}}{[1 + 10^{(pH-pKa)}]} \quad (1)$$

Experimental determination of the E_w^T and the pH for emulsions of known PGE_1^T would enable the PGE_1^I to be estimated if the pK_a, V_o, V_w, and K_d were known. The partition coefficient (K_d) was experimentally determined to be approximately 0.7. The pK_a of the PGE_1 has been estimated to be approximately 4.95 (12). The V_o and V_w were controlled during the manufacture to be 10.8 and 87.3%, respectively.

A direct and simple method for the determination of unbound PGE_1 in emulsion formulations is the ultrafiltration method. Ultrafiltration has been widely applied in the measurement of monomer–micelle equilibria (9), drug distribution in micellar systems (10), and the distribution of preservatives in oil-in-water emulsions (11). Since the particle size of the emulsion droplets was approximately 0.11 μm, a membrane with a molecular weight cutoff of approximately 10,000 daltons was considered adequate to separate the oil and aqueous phases. Analysis of the separated aqueous phase for PGE_1 allowed the estimation of E_w^T.

Prior to the use of this technique to determine the phase distribution of PGE_1, the ultrafiltration technique required validation. Membrane adsorption and rejection had to be accounted for in order to measure accurately aqueous concentrations of PGE_1. The ultrafiltration membranes (UF) which were chosen were polysulfone media (YM series, Amicon) specifically selected for their exceptionally low nonspecific binding. The effects of membrane binding and rejection of PGE_1 were studied by ultrafiltering solutions of varying concentrations of PGE_1 which were either completely nonionized (pH 3 buffer) or ionized (pH 7 buffer). Ionized PGE_1 (pH 7) was ultrafiltered at concentrations of 5 and 85 μg/ml. Nonionized PGE_1 was ultrafiltered at solution concentrations of 5 and 25 μg/ml. Recovery curves for PGE_1 from aqueous solutions at various concentrations and pH's are shown in Fig. 1. The membrane appears to be nearly saturated after approximately 5% of the total volume has been filtered as evident in the leveling off of the curves. The percentage recovery was 95–97 and 92–97% of theoretical, at pH 7 and pH 3, respectively, indicating that rejection was small but not negligible. Based on these rejection data, ultrafiltration data for the PGE_1 emulsion formulations required only a slight correction provided that at least 5% of the total volume was filtered to saturate the membrane.

A 200 μg/ml concentration of PGE_1 in the fat emulsion

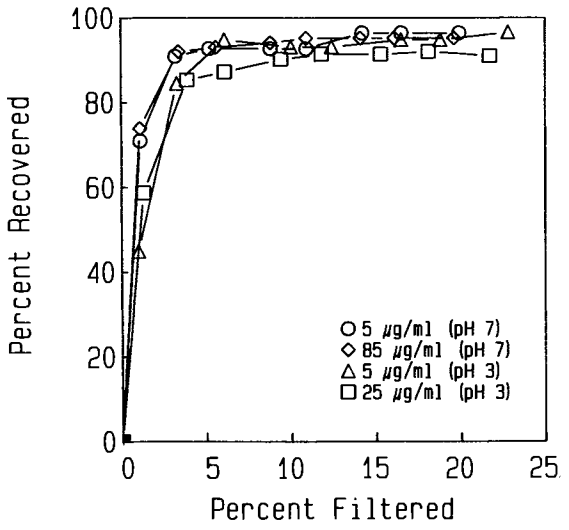

Fig. 1. Recovery curves for PGE_1 from aqueous solutions at various concentrations and pH's from ultrafiltration experiments.

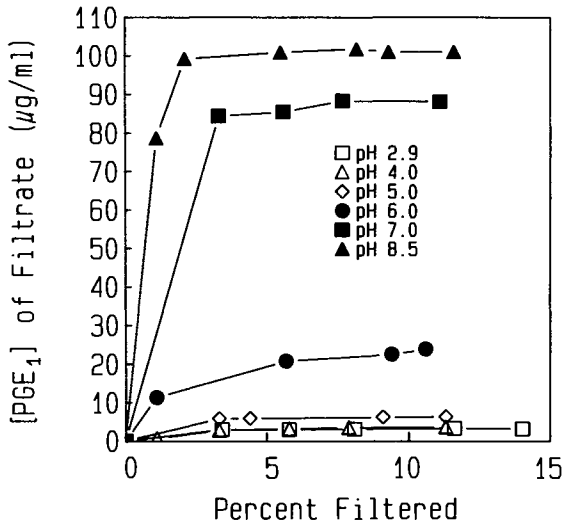

Fig. 2. PGE_1 concentration in filtrate after ultrafiltration of the 200 μg/ml PGE_1 emulsion formulation at variable pH's at 35°C.

was chosen for these experiments. This concentration enabled a sufficient assay sensitivity to be obtained for the PGE_1 HPLC assay. This concentration and the temperature of 35°C were also selected because these parameters were convenient for ensuing kinetic experiments which will be reported in a subsequent paper. Information collected at the 200 μg/ml concentration, which is still well below the critical micelle concentration (CMC) (8,12), should be either directly applicable or extrapolatable to lower concentrations such as the 5 μg/ml concentrations employed in emulsions tested clinically (1–5).

The visual appearance of the filtrate was a clear liquid, in contrast to the milky white liquid of the unfiltered emulsion. Photon correlation spectroscopy measurements with the Nicomp particle size analyzer could detect no particles in the filtrate. These data support the premise that the oil droplets are removed by the filter and that only the aqueous phase passes through the filter. Figure 2 displays the results of the ultrafiltration of PGE_1 in emulsion formulations at various pH values. The point where the filtrate concentration became constant was used as the appropriate aqueous phase concentration. Depending on the pH and concentration, the data were corrected slightly for the percentage of rejection observed in filtering aqueous solutions. None of the data were corrected by more than 8% due to membrane rejection. Using these data and Eq. (1), the distribution of PGE_1 in the three phases as a function of the pH was calculated. This distribution profile is shown in Fig. 3. The oil/water partition coefficient for PGE_1 (nonionized) was sufficiently low (approximately 0.7) that, for the phase volume ratio of 0.1, the calculated amount of PGE_1 in the oil phase was less than 1% and was considered insignificant. Therefore, only the relative amounts of PGE_1 in the aqueous phase or at the interface are plotted. It is obvious from Fig. 3 that interfacial PGE_1 predominated over most of the pH range studied.

Since both the nonionized and the ionized forms of PGE_1 are surface active, both species can exist at the interface. It is possible to obtain estimates of the relative distribution coefficients of the ionized and nonionized PGE_1 between the interface and the aqueous phase using the data already generated. These estimates assume that (a) the distribution of solute into the bulk oil phase is insignificant and (b) the volume of the interfacial layer, V_i, is approximately equal to the volume of the surfactant (lecithin). The distribution coefficients of the neutral and ionized forms of PGE_1 between the interface and the aqueous phase, K_d° and K_d^-, are then defined as

$$K_d^\circ = E_i^\circ / E_w^\circ \quad (2)$$

$$K_d^- = E_i^- / E_w^- \quad (3)$$

where E_i° and E_i^- are the concentrations of nonionized and ionized PGE_1 at the interface, respectively, and E_w° and E_w^- are the concentrations of nonionized and ionized PGE_1 in the aqueous phase, respectively.

The fraction of PGE_1 at the interface, f_i, or in the

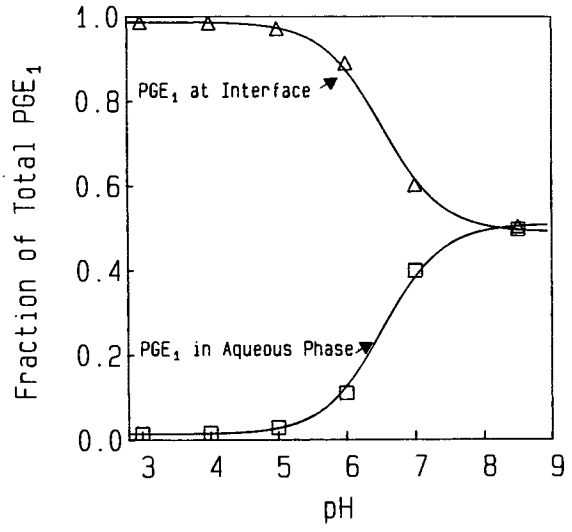

Fig. 3. Distribution of PGE_1 in the interfacial and aqueous phases in the 200 μg/ml PGE_1 emulsion as a function of pH at 35°C.

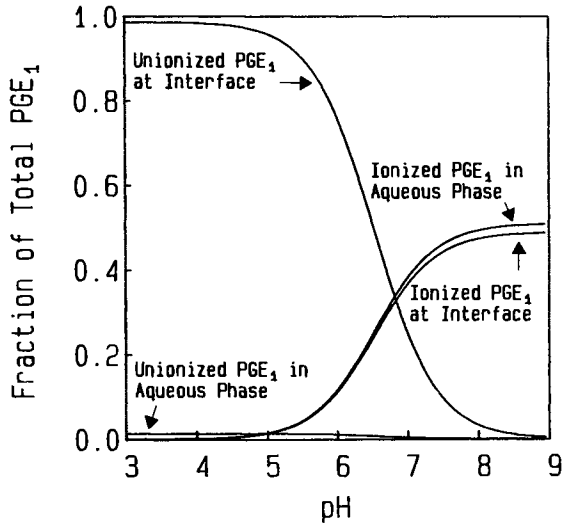

Fig. 4. Calculated distribution of ionized and nonionized PGE_1 species in the interfacial and aqueous phases in the 200 μg/ml PGE_1 emulsion as a function of pH at 35°C.

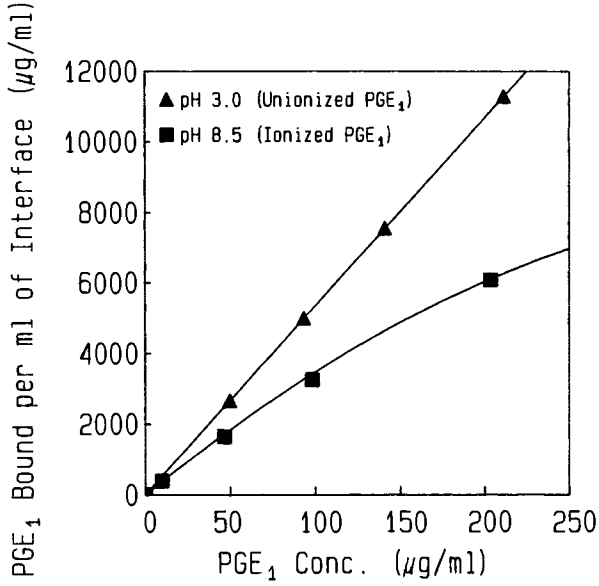

Fig. 6. Modified Langmuir adsorption isotherm for binding of both ionized and nonionized PGE_1 to the emulsion interface at 35°C.

aqueous phase, f_{aq} ($= 1 - f_i$), can be calculated in terms of the above distribution coefficients using Eq. (4):

$$f_i = \frac{(K_d^{\circ}H^+ + K_d^-K_a)V_i}{(K_d^{\circ}H^+ + K_d^-K_a)V_i + (H^+ + K_a)V_w} \quad (4)$$

Equation (4) was fitted by least-squares analysis to the data in Fig. 3 describing the PGE_1 distribution at the interface as a function of the pH to obtain estimates of K_d° and K_d^-. The fit (solid line) to the actual data points is excellent. The calculated values of K_d° and the K_d^- were found to be 3481 ± 1158 (95% CI) and 45.3 ± 7.5 (95% CI), respectively.

The use of similar relationships also enabled distribution curves to be generated for the various charged and uncharged PGE_1 species present in the phases as a function of the pH. Figure 4 contains the calculated distribution profile for the various PGE_1 species as a function of the pH. The apparent pK_a of PGE_1 at the interface, estimated from the data in Fig. 4, is 6.8. The higher pK_a for interfacially bound PGE_1 is consistent with the observation that the interface is a less favorable environment for the anionic form, due at least in part to charge repulsion at the interface.

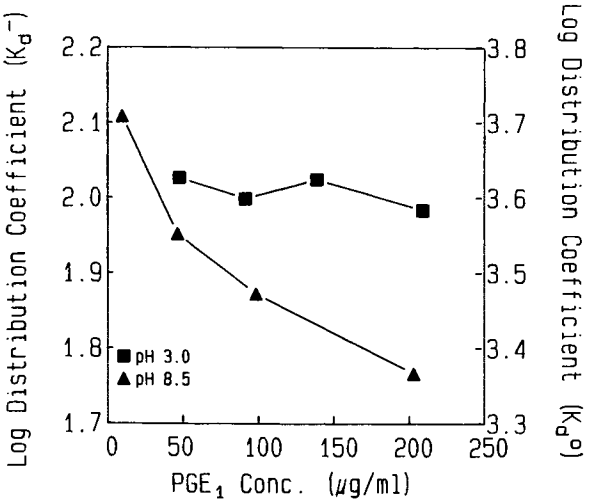

Fig. 5. Dependence of the PGE_1 concentration on the distribution coefficient for both ionized and nonionized PGE_1 between the interface and the aqueous phases at 35°C. Left-hand axis, pH 8.5; right-hand axis, pH 3.0.

An important consideration in the use of the derived distribution coefficients at 200 μg/ml to the clinically used 5 μg/ml concentration is the effect of variable PGE_1 concentrations on the distribution coefficients. This effect was evaluated by determining the distribution coefficients at both a low pH (3.0) for the nonionized PGE_1 and a high pH (8.5) for the ionized PGE_1 while varying the PGE_1 concentration. Figure 5 illustrates the results of this work. At a low pH, the distribution coefficient of unionized PGE_1 (K_d°) is independent of the drug concentration. However, at pH 8.5, where the ionized PGE_1 species predominates, the distribution coefficient (K_d^-) is dependent on the drug concentration. The data at both low and high pH levels were fitted satisfactorily with a modified Langmuir isotherm as shown below:

$$A_i = y_m b(E_T - A_iV_i)/[V_w + b(E_T - A_iV_i)] \quad (5)$$

where E_T equals the PGE_1 concentration, A_i equals the amount of PGE_1 bound to the interface per milliliter of interface, y_m reflects the capacity of the surface, and b is the ratio of rate constants for adsorption and desorption. A plot of the data according to the modified Langmuir adsorption isotherm equation shown above is illustrated in Fig. 6. The linear response shown for the nonionized PGE_1 illustrates again that the binding to the interface is independent of the nonionized PGE_1 concentration. The nonlinear response for the ionized PGE_1 also again illustrates that the binding to the interface is dependent on the ionized PGE_1 concentration. The data fit yields a $y_m = 12{,}000 \pm 2000$ and $b = 0.0100 \pm 0.0028$ for the ionized PGE_1 and a $y_m = 63{,}000 \pm 21{,}000$ and $b = 0.073 \pm 0.029$ for the nonionized PGE_1. The products

(by_m = 120 for the ionized PGE_1 and by_m = 4600 for the nonionized PGE_1) are estimates of the distribution coefficients for both the ionized and the nonionized forms of PGE_1 at high dilutions. The decrease in K_d^- with increasing drug concentrations may be the result of the increase in the negative charge at the interface, as the negatively charged PGE_1 binds to the interface and makes the binding of the additional ionized drug more difficult.

The data presented in this report should be quite useful in accounting for the degradation of PGE_1 in fat emulsions as a function of pH and drug concentration. Since prostaglandins have much improved stability in aprotic solvents (12), PGE_1 might be expected to exhibit improved stability in lipid emulsion formulations. However, this study has shown that the fraction of PGE_1 residing in the interior of the oil droplets is very small and that most of the compound, particularly at low pH's where stability is likely to be optimal, resides at the oil/water interface. The reactivity of surface-bound PGE_1 may differ from that in the oil interior. A subsequent report will address the kinetics of PGE_1 degradation in lipid emulsion systems.

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REFERENCES

1. Y. Mizushima, A. Yanagawa, and K. Hoshi. *J. Pharm. Pharmacol.* 35:666–667 (1983).
2. Y. Mizushima, Y. Wada, Y. Etoh, and K. Watanabe. *J. Pharm. Pharmacol.* 35:398–399 (1983).
3. Y. Mizushima. *Drugs Exp. Clin. Res.* XI(9):595–600 (1985).
4. S. Otomo, Y. Mizushima, H. Aihara, K. Yokoyama, M. Watanabe, and A. Yanagawa. *Drugs Exp. Clin. Res.* XI(9):627–631 (1985).
5. K. Hoshi, Y. Mizushima, S. Kiyokawa, and A. Yanagawa. *Drugs Exp. Clin. Res.* XII(8):681–685 (1986).
6. A. J. Repta. In D. D. Bremer and P. Speiser (eds.), *Topics in Pharmaceutical Sciences,* Elsevier/North-Holland, Amsterdam, 1981, pp. 131–151.
7. A.-A. A. El-Sayed and A. J. Repta. *Int. J. Pharm.* 13:303–312 (1983).
8. M. C. R. Johnson and L. Saunders. *Biochim. Biophys. Acta* 218:543–544 (1970).
9. I. W. Osborne-Lee, R. S. Schechter, and W. H. Wade. *J. Colloid Interface Sci.* 94:179 (1983).
10. M. Nakagaki and S. Yokoyama. *Chem. Pharm. Bull.* 33:2654–2662 (1985).
11. T. Shimamoto, Y. Ogawa, and N. Ohkura. *Chem. Pharm. Bull.* 21:316–322 (1973).
12. R. G. Stehle. *Methods Enzymol.* 86:436–458 (1982).