CHAPTER IV

RESULTS AND DISCUSSION

1.1 Determination of Percent Labeled Amount of Piroxicam

The purpose of this part of study was to standardize the topical piroxicam gel products with respect to the percent labeled amount prior to further *in vitro* and *in vivo* studies. The assay results are shown in Table 1. From this table, it is apparent that all the six products contained an equal amount of piroxicam, with the percent labeled amount for each product within the range of 90.0-110.0%. Therefore, they could be used for further experiments since any differences found in the release or permeation characteristics of these products would not be due to the difference in their initial amount of piroxicam.

1.2 *In Vitro* Release Studies of Piroxicam through Synthetic Membrane

These experiments were conducted to evaluate the *in vitro* release characteristics of six piroxicam gel products using the diffusion apparatus and synthetic membranes. The six products (A, B, C, D, E and F) which previously passed the test for percent labeled amount were further subjected to this part of the study.

The physiological availability of a topically applied drug depends on both the release of drug from the vehicle as well as its permeability through

Table 1 : Percent labeled amount of piroxicam gel products

Product	Percent labeled amount		
	Individual values		Mean (n = 2)
A	100.5	101.7	101.1
B	106.4	104.6	105.5
C	100.9	100.0	100.5
D	102.0	104.0	103.0
E	105.0	107.0	106.0
F	108.2	108.8	108.5

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the skin. In addition, release rate of the drug from topical preparations directly depends on the physicochemical properties of the vehicle and the drug itself. Therefore, the type of membrane utilized for the drug release study should have a minimal influence on the drug release characteristics in order to accurately observe the effect of the vehicle and drug properties. The cellulose acetate membranes which were used in this study to evaluate piroxicam release from the gel products are porous membranes (pore size = 0.45 μm). Therefore, they are not expected to produce any significant barrier effect against the drug release since the drug molecules should pass freely through the pores of this membrane. It is a hydrophilic, porous membrane, commonly used in the *in vitro* drug release studies to prevent dispersion of the formulation into the receptor fluid. As a result, it mainly acts as a support and not as a diffusion barrier.

Theoretical Background of *In Vitro* Release from Vehicle.

There are several identifiable processes that form the basic of the release kinetics of drugs from the vehicle, particularly when the release is rate-limiting. Higuchi depicted the situation in which the vehicle is initially saturated with the solute, with excess solute uniformly suspended as tiny particles (Addick and Weiner, 1987). The important assumption for derivation of the time dependency of the release is that Q (the total concentration of drug, μg/cm^3) is much greater than C_s (the solubility of drug in the vehicle, μg/cm^3).

The equation describing the release of a solute is

$$M_t = [2DC_s (Q-C_s t/2)]^{1/2}$$

where M_t is the amount of drug released per unit area (μg/cm^3), D is diffusivity (cm^2/sec) of the drug in the vehicle. And the rate of release is

$$\frac{dM_t}{dt} = \frac{[DC_s(2Q-C_s)/t]^{1/2}}{2}$$

when $Q >> C_s$, the amount of drug released into a sink bears the following relationship to time :

$$M_t = [2DC_s Qt]^{1/2}$$

This equation predicts that a plot of amount of drug release versus the square root of time should be linear and that

$$\frac{dM}{dt} = [DC_s Q/2t]^{1/2}$$

This equation predicts that the rate of drug release is proportional to the reciprocal of the square root of time.

According to the above model where the membrane is not rate-limiting, the mass of drug released from the formulation through a porous membrane should be proportional to the square root of time. As the drug molecules partition from the formulation into the receptor, the remaining drug in the vehicle matrix must reequilibrate into the new volume, leading to a decrease in total drug concentration in the vehicle. As a result, the plot of the cumulative amount of drug in the receptor fluid and the square root of

time should be linear with the slope representing the release rate (amount of drug released per square root of time).

The release profiles of piroxicam from the six commercial products through cellulose acetate membrane are presented in Figure 14. The numerical data are also given in Table 2. The profiles were plotted between the cumulative amount of drug and square root of time according to the above model. As can be seen from this figure, such plots give relatively linear relationships with the regression coefficients (r^2) greater than 0.99 in most products (Appendix II). On the contrary, Figure 15 show the zero order plots between cumulative amount of piroxicam released as a function of time. It can be seen from these figures that the lines were not linear in all products. These results thus support the use of porous synthetic membrane like cellulose acetate for evaluating piroxicam release from the topical gels as well as the application of Higuchi square root of time plot to the treatment of data.

Since the plots in Figure 14 demonstrate linear relationship over the six hours sampling period, it can be implied that the vehicle was controlling the drug release according to the Higuchi equation where the drug was being released from the matrix and that passage of the drug through the porous membrane occurred freely. The lack of lag time in all plots also support this finding. Actually, the zero order plots in Figure 15 even demonstrate the initial burst of drug released into the receptor fluid, with the slope being maximum in the initial period and gradually decreasing with the cellulose

Table 2 : Diffusion data for six brands of Piroxicam gels released through cellulose acetate membrane

Time	Amount of Piroxicam Released* (μg/cm^2)					
(hr)	A	B	C	D	E	F
0.50	73.945 ± 6.680	235.579 ± 33.330	192.590 ± 2.838	93.536 ± 7.427	120.524 ± 34.680	195.289 ± 19.359
1.00	162.409 ± 16.993	381.894 ± 55.108	297.565 ± 10.514	177.727 ± 12.727	224.090 ± 29.046	300.623 ± 27.412
1.50	258.926 ± 5.013	509.364 ± 63.938	379.555 ± 22.609	251.587 ± 17.892	282.764 ± 32.049	393.614 ± 28.577
2.00	312.109 ± 7.274	607.196 ± 68.660	445.007 ± 29.907	312.691 ± 22.373	350.128 ± 31.653	473.160 ± 23.556
2.50	342.290 ± 29.857	704.814 ± 71.604	521.898 ± 40.620	368.270 ± 28.312	415.228 ± 26.900	543.200 ± 33.482
3.00	398.386 ± 25.375	786.822 ± 75.029	588.969 ± 34.312	417.685 ± 36.223	475.419 ± 23.116	619.725 ± 33.471
3.50	442.249 ± 31.355	870.313 ± 78.476	653.709 ± 29.457	470.923 ± 40.344	525.316 ± 25.121	693.730 ± 29.548
4.00	487.568 ± 37.636	973.674 ± 104.052	722.537 ± 27.862	522.722 ± 44.355	571.631 ± 25.942	748.284 ± 32.304
5.00	552.204 ± 46.405	1097.645 ± 94.120	828.958 ± 21.844	607.466 ± 51.825	664.287 ± 26.134	860.008 ± 25.614
6.00	615.997 ± 55.867	1223.502 ± 102.063	945.995 ± 22.331	686.022 ± 59.312	749.311 ± 28.447	957.851 ± 21.308

*n = 3, mean ± SD

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Figure 14: Release profiles of six piroxicam gel products released through cellulose acetate membrane (Value = mean ± SD, n = 3)

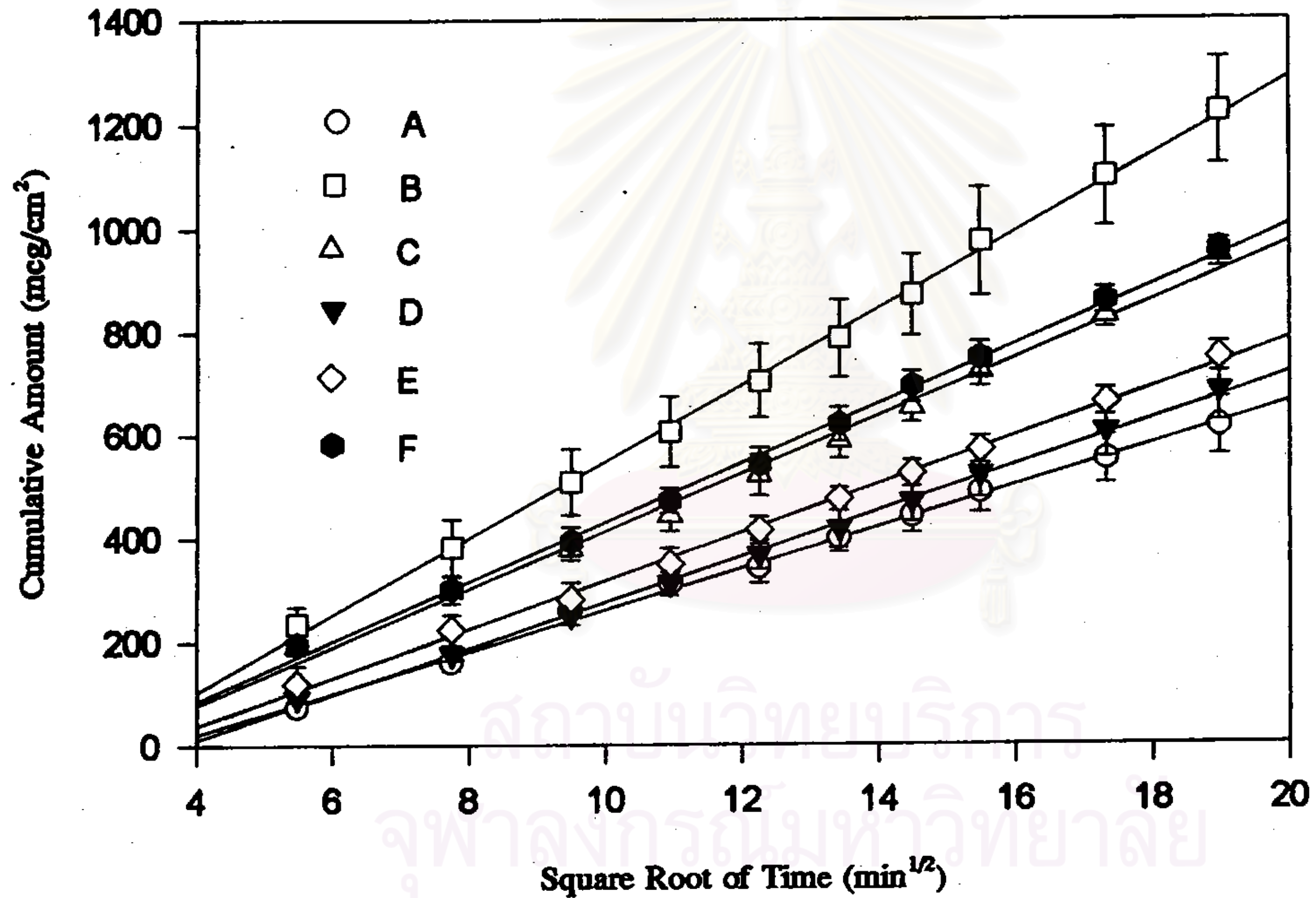

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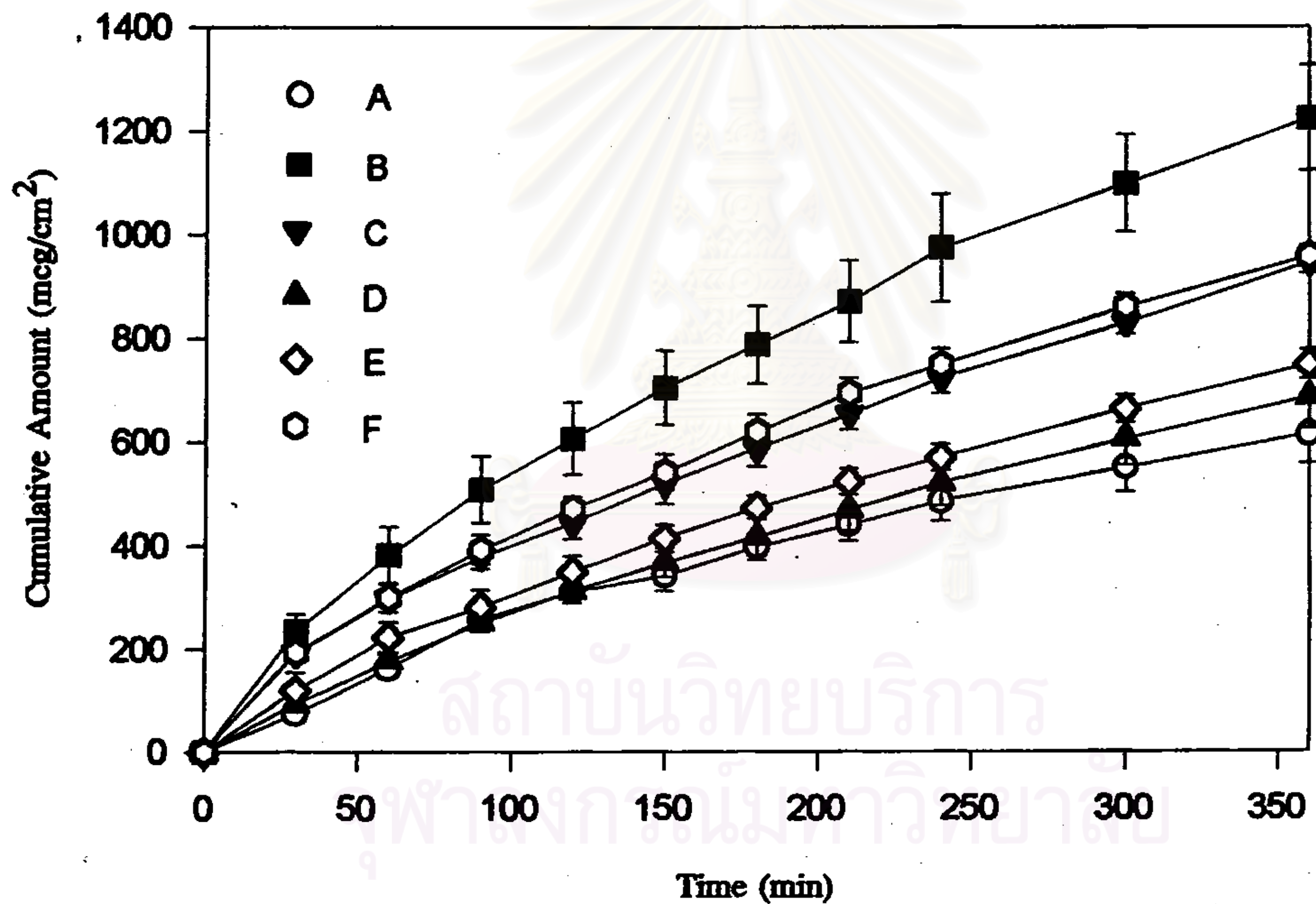
Figure 15: Diffusion profiles of six piroxicam gel products released through cellulose acetate membrane (Value = mean ± SD, n = 3)



acetate membrane. The averages of release rate and cumulative amount from triplicate runs of the six products are also shown in Table 3 for cellulose acetate membrane and their calculations are provided in Appendix II.

The donor chamber of each diffusion cell contained 1.5 g of piroxicam gel product. This would give an excessive amount of drug to be released and avoid depletion of the drug during study, then maximizing the concentration gradient across the membrane. On the other hand, the receptor fluid consisted of pH 7.4 isotonic phosphate buffer. It has been reported that the approximate solubility of piroxicam as determined by Herzfeldt and Kummel (1983) was 2 - 3 mg/ 100 ml at pH 2.0; 7.6 mg/ 100 ml at pH 6.0; 57 mg/ 100 ml at pH 7.0; and 103 mg/ 100 ml at pH 7.5. Since piroxicam is a free acid with pKa about 5.3 (Herzfeldt & Kummel, 1983), the drug would be essentially fully ionized in this buffer. As a result, the pH 7.4 isotonic phosphate buffer was selected as the receptor fluid so as to facilitate the dissolution and diffusion process.

After each release study, the samples were kept in the refrigerator at 4°C and analyzed within one week for piroxicam contents. Previous HPLC analysis has shown that there was no sign of degradation during this storage period.

Table 3 : Comparison of average cumulative amount and release rate of six piroxicam gel products through cellulose acetate membrane at 6 hr

Product	Cumulative amount release* ($\mu g/cm^2$)	%CV	Release rate* ($\mu g/cm^2.min^{1/2}$)	%CV
A	615.997 ± 55.867	9.07	39.98 ± 4.64	11.60
B	1223.502 ± 102.063	8.34	74.00 ± 4.83	6.53
C	945.995 ± 22.331	2.36	55.79 ± 1.99	3.57
D	686.022 ± 59.312	8.64	44.25 ± 4.13	9.33
E	749.311 ± 28.447	3.80	46.64 ± 2.86	6.13
F	957.851 ± 21.308	2.22	57.48 ± 0.31	0.54

*n=3, mean ± SD

Table 4 : Result from Duncan's test of *in vitro* release study

Cellulose acetate membrane	F-Value	P-Value	Result
1. Cumulative amount released at 6 hr ($\mu g/cm^2$)	32.0246	p = 0.0001	A < D < E < C < F < B *(p < 0.05)
2. Release rate ($\mu g/cm^2.min^{1/2}$)	24.4515	p = 0.0001	A < D < E < C < F < B *(p < 0.05)

*significant at α = 0.05

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1.2.1 Statistical Analysis of Piroxicam Release through Cellulose Acetate Membrane

Analysis of variance (ANOVA) was then applied to the data obtained from the release studies at 5% significance level. From the ANOVA tables (Appendix III), it is obvious that there was a significant difference in the cumulative amount of piroxicam released from the six products through cellulose acetate membrane at 6 hour ($p < 0.05$). A multiple range test (Duncan's test) was further applied to these data at the same significance level in an attempt to rank the six products with respect to their release characteristics. The results are shown in Table 4. As can be seen from this table, the ranking of the cumulative amount of drug released through this membrane, in an increasing order, was $\underline{A < D} < E < \underline{C < F} < B$. The three lines underneath the letters A and D, D and E and letter C and F signify that there was no significance difference between products on each line ($p > 0.05$). For example, although product E released piroxicam to a greater extent than D, product D released greater extent than A and product F released greater than C, differences were not significant.

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However, product B released the greatest amount of piroxicam after 6 hours and the value differed significantly from others. Products C and F also released piroxicam to a significantly greater extent than products A, D and E. Based on the results observed with the extent of drug release, the six products can be roughly classified into four groups, i.e. a group with the greatest amount of drug released (Product B 1223.50 ± 102.06 $\mu g/cm^2$), the great amount of drug released (Product F 957.85 ± 21.31 $\mu g/cm^2$ and Product

C 946.00 ± 22.33 $\mu g/cm^2$), the moderate amount of drug released (Product E 749.31 ± 28.45 $\mu g/cm^2$; Product D 686.02 ± 59.31 $\mu g/cm^2$) and the smallest amount of drug released (Product A 616.00 ± 55.87 $\mu g/cm^2$).

When ANOVA was applied to the release rate data from the same membrane, there was significant differences at 5% level (Table 4, Appendix III). The ranking order of the release rate was $\underline{A < D < E} < \underline{C < F} < B$ (the line underneath the letters signify non-significance). Based on the values of their release rate, the six products can also be roughly classified into three groups. For example, product A, D and E had similar release rates (39.98 ± 4.64, 44.25 ± 4.13 and 46.64 ± 2.86 $\mu g/cm^2.min^{1/2}$) but released piroxicam significantly slower than product C and F (55.79 ± 1.99 and 57.48 ± 0.31 $\mu g/cm^2.min^{1/2}$). The release rate of product B, on the other hand, (74.00 ± 4.83 $\mu g/cm^2.min^{1/2}$) was the fastest. As a result, the classification of products may be assumed, i.e. a group with fast release rate (B), a group with moderate release rate (C and F) and a group with slow release rate (A, D and E).

One product of each groups was then selected as a representative for further *in vitro* permeation and *in vivo* studies. So the products B, C and D were selected to represent the fast, moderate and slow release groups, respectively, in comparison to the product A which is an innovator piroxicam gel product.

1.3 *In Vitro* Permeation Studies through New Born Pig Skin

Since the possible rate limiting step of the overall percutaneous absorption could be either the release of the drug from the vehicle or the penetration of the drug through the skin barrier, investigation of the *in vitro* permeation of piroxicam through the skin membrane was also carried out in addition to the *in vitro* release studies. For topical products, permeability of the drug through the skin may be the most limiting factor which could lead to the differences in bioavailability and effectiveness.

The new born pig skin was selected as a model membrane for the *in vitro* permeation studies because in the previous report, statistically significant correlation was found between the percutaneous penetration of 10 compounds on whole pig skin *in vitro* and reported values for human skin *in vivo* (Hawkins and Reifenrath, 1986). There is a study compare clinical results with absorption studies in rats, rabbits, miniature swine and rhesus monkey. The topical antifungal agent Haloprogin penetrates well in the rat and rabbit but to a lesser degree in the pig and man , where results are similar. The rat and rabbit absorb about 25 to 30% of an applied cortisone dose, but little passes into the pig and man. The skin of miniature swine and rhesus monkeys behave somewhat similarly to that of man. There is evidence that porcine skin shares some histochemical, anatomic and penetration feature with human skin. Turning from the *in vivo* to the *in vitro* situation, several publications present data which rank skin permeability in different species. Although these studies use different compounds and different anatomic sites for skin samples, in general it appears that the skins of laboratory animals

such as guinea pig, rat and rabbit are more permeable than human skin. Skin from the monkey and the pig is closest to that of man. It may summarize the comparative studies of skin penetration *in vivo* and conclude that in general the skins of the monkey and the pig are most like that of man. The Guidelines for Preclinical and Clinical Testing of New Medicinal Products, published by the Association of the British Pharmaceutical Industry, recommend the use of piglets on the basis that their skin is similar in many ways to human skin. In addition, the permeability of pig and monkey skins approximates to that of man (Barry, 1983).

The studies were evaluated four possible models for predicting skin penetration in man: the human-skin-grafted congenitally (nude) mouse ("man-mouse"), the pig-skin-grafted nude mouse ("pig-mouse"), the hairless dog and the weanling Yorkshire pig. They found the significant correlation between human absorption and penetration in the weanling pig. So the weanling pig (although perhaps not the most convenient and readily available species either) offer new, alternative models of much promise and potential (Huang 1987).

The *in vitro* permeation study utilized pig skin specimens of a new-born pig which were obtained from pig farm in Nakhon Pathom. The abdominal skin was immediately collected after the newly born pigs were died and kept in the freezer until the experiments. Before use, the specimens were thawed at room temperature. Then, they were cut into small round pieces with the size slightly larger than the diffusion cell inner diameter. The

membrane was subsequently immersed in pH 7.4 isotonic phosphate buffer for 3 hr before mounting onto the diffusion apparatus to allow for hydration and swelling.

Four gel products that were selected from the previous *in vitro* release study were used for penetration experiments. The average amounts of the four gel products penetrating through the new born pig skin at various times during the 24 hr experiments are shown in Table 5. The average cumulative amounts of drug penetrated at 24 hr as well as the steady-state fluxes are also summarized in Table 6 for each product. The steady flux was obtained from the slope after linear regression of the terminal portion of the plot of the cumulative amount permeated (ng/cm^2) versus time. Individual diffusion data, determination of the fluxes and permeation profile for each product are provided in Appendix IV. From these data, the calculated regression analysis gave r^2 values which were greater than 0.98 in most of the experiments, indicating the establishment of steady-state diffusion through this skin model according to Fick's laws of diffusion (Martin et al, 1993).

The process of percutaneous absorption is usually a passive diffusion in which the matter moves from the higher concentration to the lower concentration. The diffusion of drug may therefore be expressed by Fick's first law.

Table 5 : Permeation data for four brands of Piroxicam gels permeated through new born pig skin

Time (hr)	Amount of Piroxicam Permeated* (ng/cm^2)			
	A	B	C	D
3.0	** 44.21 ± 19.45	76.12 ± 27.94	115.00 ± 17.22	236.96 ± 52.36
6.0	95.88 ± 41.06	244.06 ± 67.97	218.79 ± 48.80	657.93 ± 145.67
9.0	185.55 ± 63.70	476.71 ± 160.54	351.75 ± 100.21	1142.00 ± 216.11
12.0	289.34 ± 94.76	701.07 ± 242.49	495.34 ± 158.67	1689.75 ± 290.66
15.0	405.40 ± 130.23	932.97 ± 326.46	684.71 ± 258.34	2295.16 ± 351.33
18.0	540.57 ± 161.73	1145.28 ± 410.28	903.46 ± 378.69	2992.99 ± 446.02
21.0	695.19 ± 202.16	1339.46 ± 487.72	1119.08 ± 512.80	3704.15 ± 480.41
24.0	858.48 ± 239.17	1544.84 ± 577.97	1365.85 ± 659.71	4501.80 ± 523.60

*n = 3, mean ± SD

**n = 2, mean ± SD

Table 6 : Comparison of average cumulative amount at 24 hr and flux of piroxicam permeation from four gel products through new born pig skin

Product	Cumulative Amount* (ng/cm^2)	%CV	Flux* ($ng/hr.cm^2$)	%CV
A	858.48 ± 239.17	27.86	44.92 ± 12.33	27.45
B	1544.84 ± 577.97	37.41	72.28 ± 28.14	38.93
C	1365.85 ± 659.71	48.30	68.19 ± 38.23	56.06
D	4501.80 ± 523.60	11.63	213.80 ± 21.92	10.25

*n = 3, mean ± SD

Table 7 : Duncan's test result on *in vitro* permeation of four piroxicam gel products through new born pig skin

New born pig skin	F-value	P-value	Result
1. Cumulative amount release (ng/cm^2)	19.7538	p = 0.0009	$\underline{A < C < B} < D$ *(p < 0.05)
2. Flux ($ng/hr.cm^2$)	16.4151	P = 0.0013	$\underline{A < C < B} < D$ *(p < 0.05)

* significant at $\alpha = 0.05$

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Fick's First Law of Diffusion

$$J = dM/S.dt$$

Where J is the flux, amount per time per unit area, $ng/hr.cm^2$

M is the cumulative amount of diffusant which passes per unit area through the membrane, ng/cm^2

S is cross sectional area, cm^2

t is time, hr

The flux in turn is proportional to the concentration gradient, dC/dx:

$$J = -D\frac{dC}{dx}$$

D is the diffusion coefficient of a penetrant, cm^2/hr

C is the concentration, ng/cm^3

x is the distance in cm of movement perpendicular to the surface of the barrier

An important condition in the diffusion is that of the steady state.

The first law of Fick may be rewritten as

$$J = \frac{DK(C_1-C_2)}{h}$$

C_1, C_2 is concentration in the doner and receptor sides, ng/cm^3, respectively

h is thickness of membrane barrier, cm

K is partition coefficient of solute between membrane and bathing solution

At the steady state in diffusion experiments, the solution in the receptor compartment is constantly removed and replaced with fresh solvent to keep the concentration at a relatively low level (or as sink condition), therefore $C_1 >> C_2$ and $C_2 \rightarrow 0$, the steady state flux (J_{ss}) can be described as below :

$$J_{ss} = PC_{ss}$$

Where J_{ss} = Steady state flux of permeation, ng/hr.cm^2 = constant

P is Permeability coefficient, cm/hr, where P = DK/h

C_{ss} is Concentration gradient across the membrane barrier at steady state, ng/cm^3, = C_1

Since the above equation was derived from Fick's first law, consistency of the data to this equation as demonstrated by the calculated regression coefficient should indicate the diffusion-controlled permeation of the drug through the new born pig skin membrane.

Permeation profiles or plots of cumulative amount of piroxicam permeated through the new born pig skin at various times are respectively shown in Figure 16 for the four products. Comparison of the pig skin data with the cellulose acetate membrane was also made and graphically shown in Figure 17-20. From these figures, it can be seen that the release and permeation of piroxicam through the new born pig skin was much slower than through the cellulose acetate membrane regardless of the products. The most rapid release of the drug was observed with hydrophilic cellulose acetate membrane. This is not unexpected since the new born pig skin is not a

Figure 16: Comparison of permeation profiles of four piroxicam gel products through new born pig skin (Value = mean ± SD, n = 3)

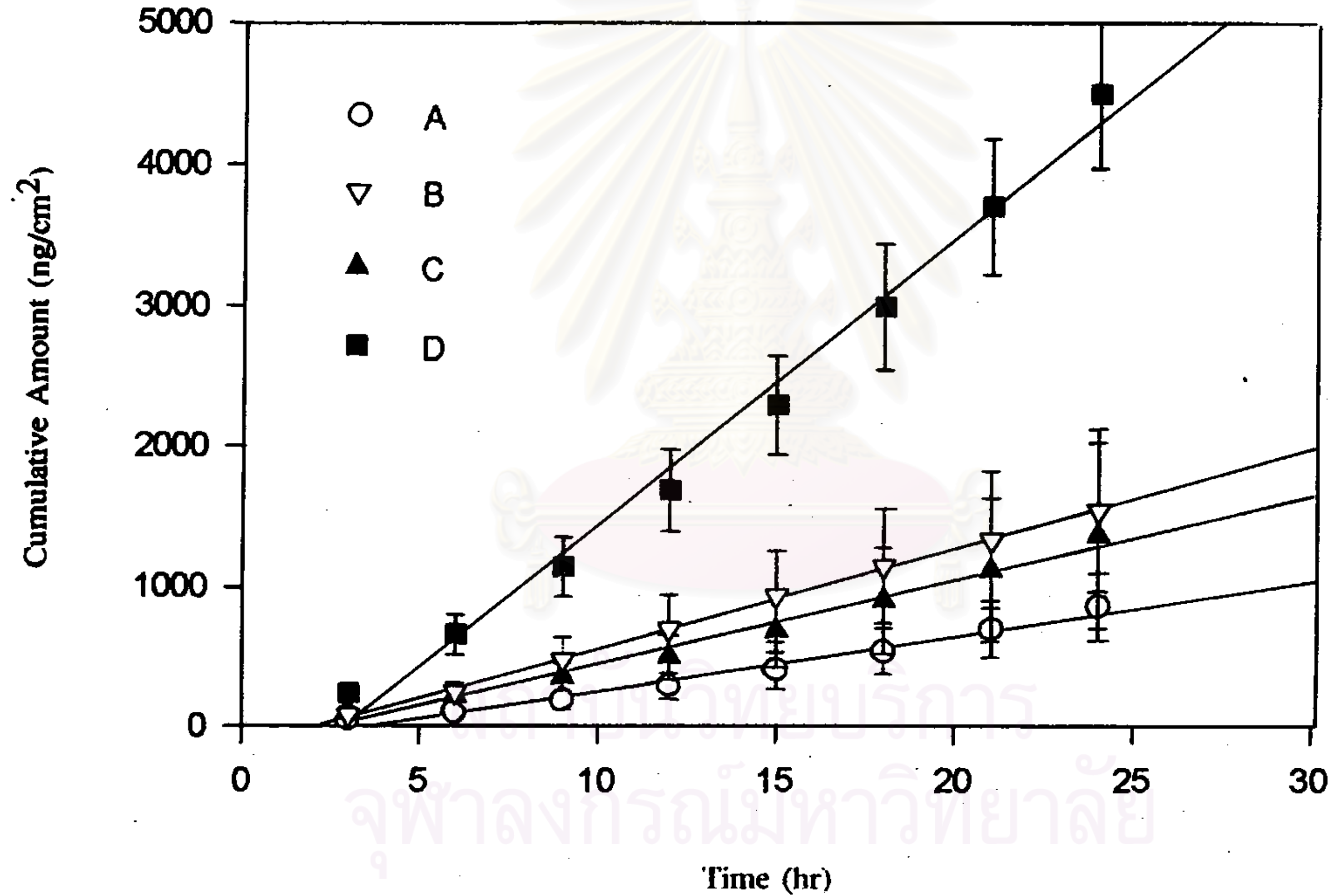

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Figure 17 : Diffusion profiles through cellulose acetate membrane and new born pig skin of product A (Value = mean ± SD, n = 3)

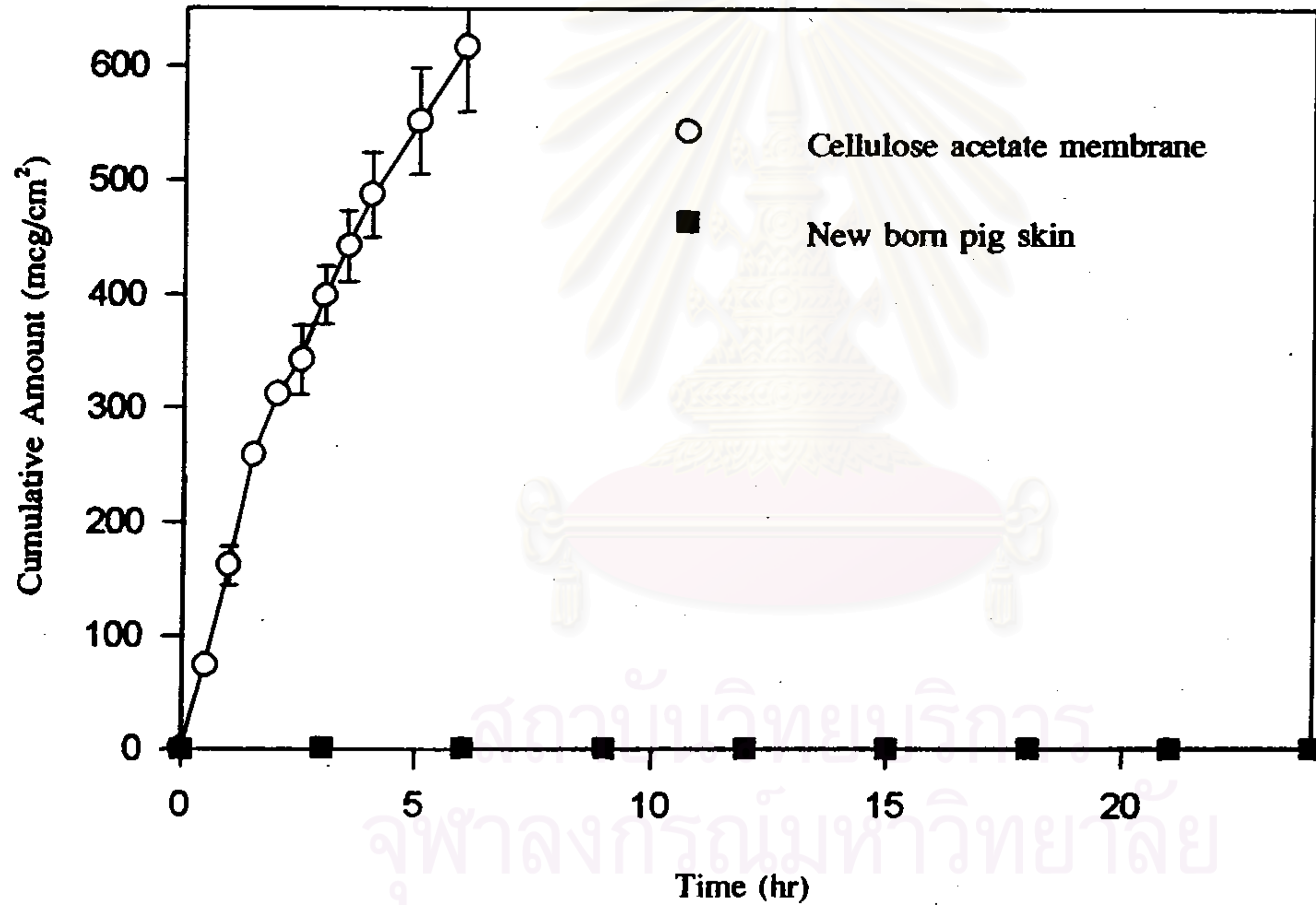

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Figure 18: Diffusion profiles through cellulose acetate membrane and new born pig skin of product B (Value = mean ± SD, n = 3)

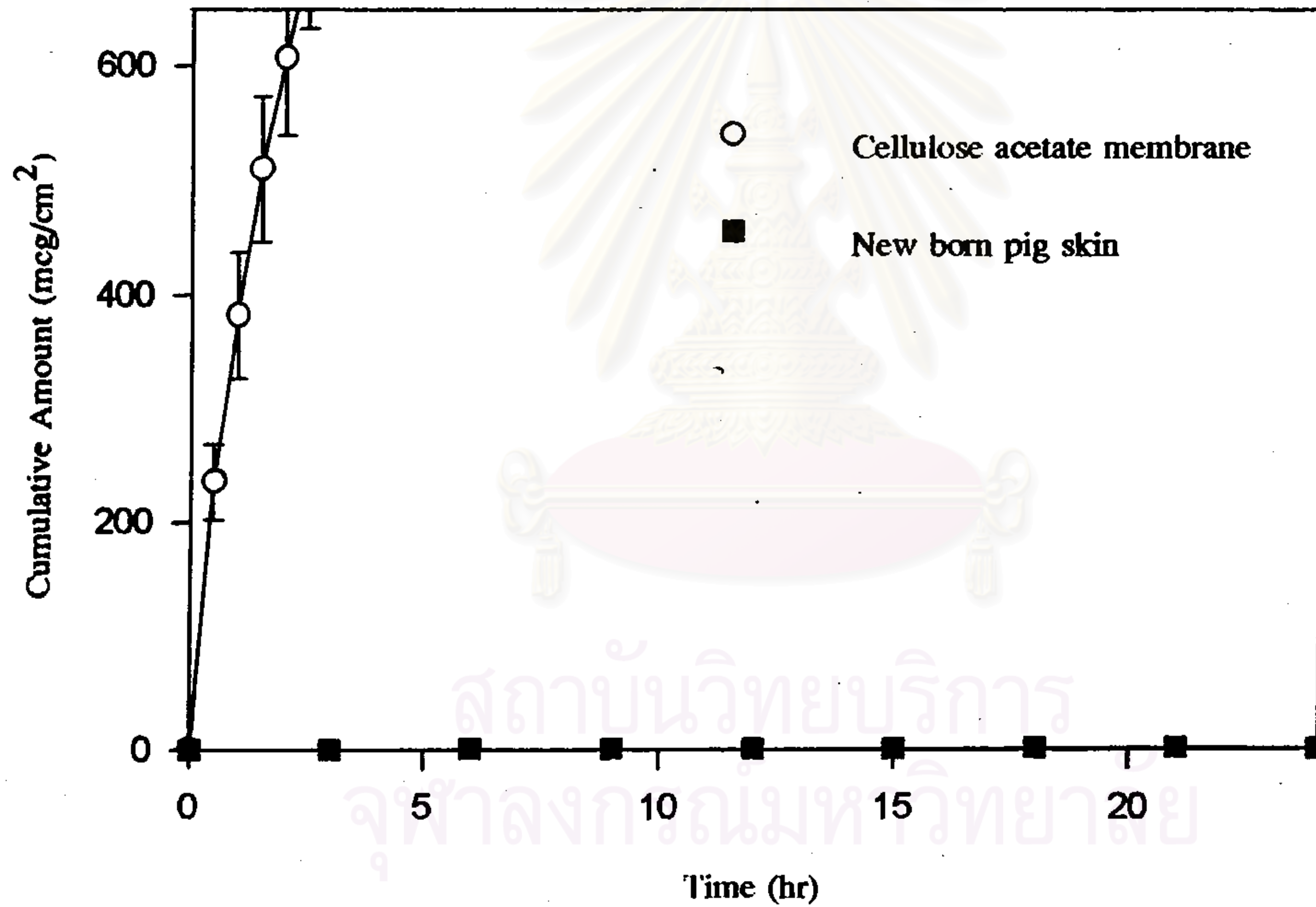

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Figure 19: Diffusion profiles through cellulose acetate membrane and new born pig skin of product C (Value = mean ± SD, n = 3)

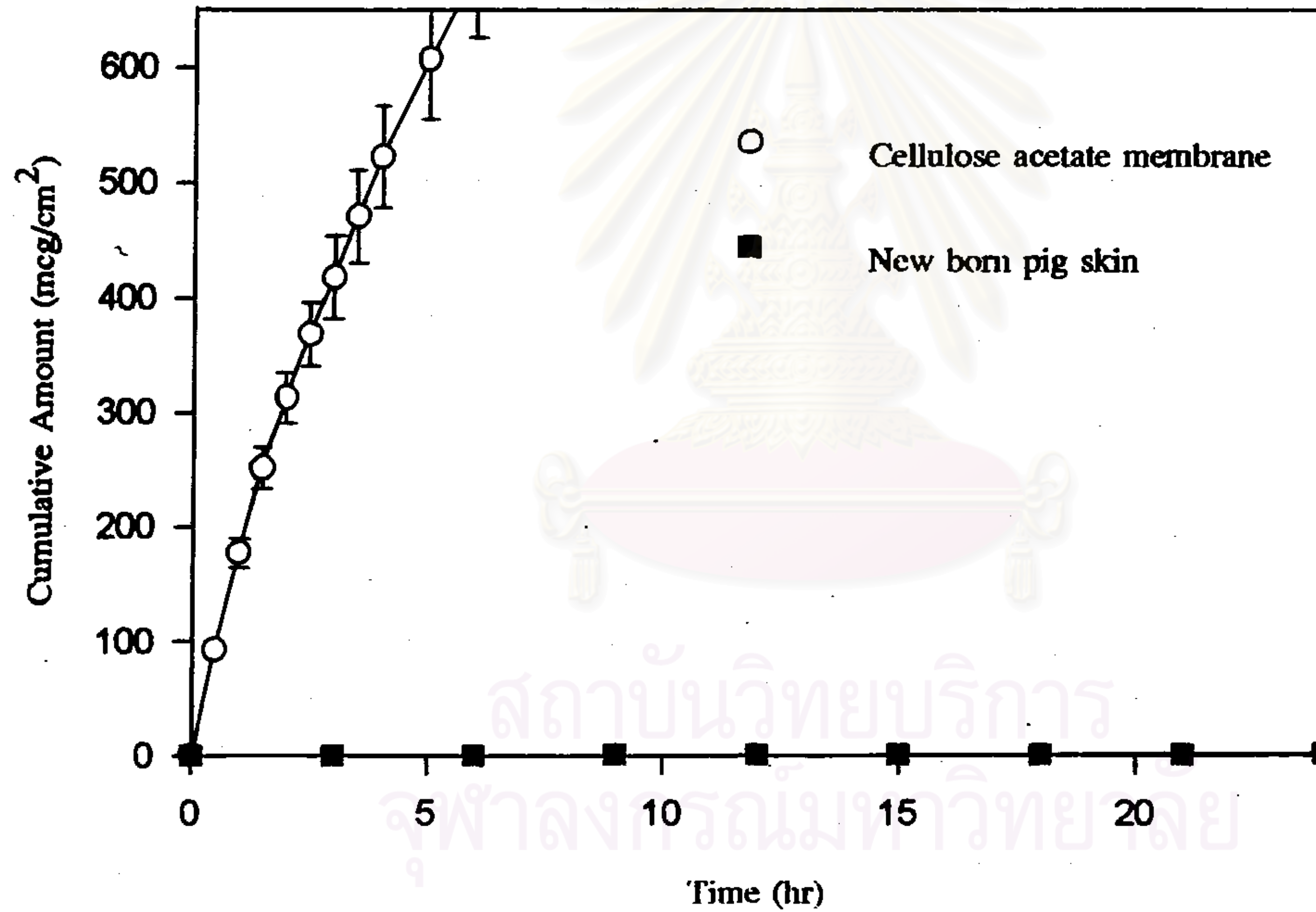

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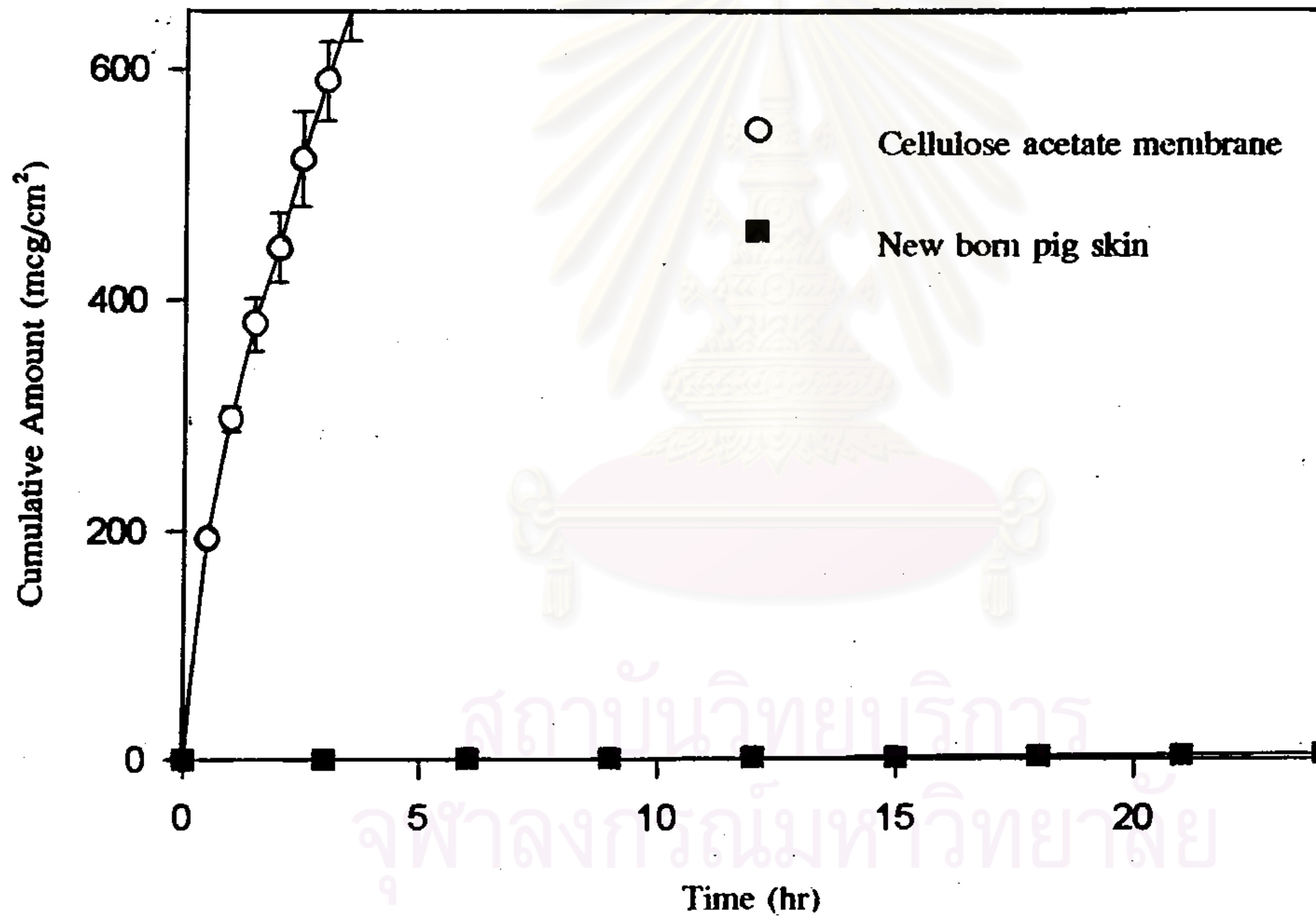
Figure 20: Diffusion profiles through cellulose acetate membrane and new born pig skin of product D (Value = mean ± SD, n = 3)



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porous membrane in contrast to the cellulose acetate membrane. Rather, it consists of heterogeneous system in which ionized species diffuse across aqueous shunt pathway and unionized species penetrate through the lipophilic barrier. On the other hand, the synthetic membrane did not play a significant barrier role against piroxicam diffusion. It mainly served as a support to prevent direct mixing of the gel base with the receptor fluid. As long as the drug can be released from the vehicle, passage of the drug into the receptor fluid can occur readily through the pores of this membrane. Drug penetration through the new born pig skin, however, was a more complex process since the membrane played a important barrier role. The overall permeation or the steady state flux was a result of two components, i.e. the release of drug from the vehicle as well as its penetration through multicomponent skin structures. Since the new born pig skin is the heterogeneous system with an average thickness of 0.026 ± 0.002 cm, it may act as an important barrier against percutaneous absorption. Penetration of the drug through this membrane thus occurred more slowly and could be the rate limiting step for the overall permeation process. This was also supported by the observed lag time of about 4 hours before the steady state flux could be achieved (Appendix V).

1.3.1 Statistical Analysis of Piroxicam Flux and Cumulative Amount Permeated through the New Born Pig Skin

ANOVA was then applied to the data obtained from the permeation studies at 5% significance level. The ANOVA results (Appendix VI) show that there were significant differences in the steady state flux and cumulative amount permeated at 24 hr among the four products. Comparison of the

permeation profiles is also given in Figure 16. The Duncan's test was further applied to these data and the results are shown in Table 7. It can be seen from this table that the ranking results, in an increasing order, for the cumulative amount permeated is A < C < B < D, and the flux is A< C < B < D. The line underneath the letter A, C and B signifies that there was no significant difference among these products. Also, product A, C and B were significantly smaller than D at 5% level with respect to the cumulative amount permeated as there was no line joining B and D. On the other hand, all four products showed similar permeation flux ($p > 0.05$) since they joined together by the same line. The statistical results from the new born pig skin were different from those previously obtained from the cellulose acetate membrane (A < D < C < B). The most likely explanation for the discrepancies in the ranking results observed between the cellulose acetate and the new born pig skin membrane was due to the barrier properties of the latter. The presence of an additional step (penetration of the drug through the rate-limiting skin barrier) in the overall permeation process across the new born pig skin could lead to the differences in the permeation characteristics of piroxicam from each product. Nevertheless, the extent to which the barrier properties of the new born pig skin modified the overall permeation of piroxicam from these products and their ranking results is not clearly known. The data only implied that penetration of piroxicam through the new born pig skin occurred at a rate which was much slower than the release of the drug from the vehicle. Therefore, further *in vivo* experiments in animals and humans should be conducted and tested for correlation with the *in vitro* release/permeation results in order to determine the most reliable method for

evaluating topical piroxicam gels. If any correlation has been found, either with the *in vitro* release or the *in vitro* permeation studies, the use of such *in vitro* tests should also have some predictive values in assessing the topical bioavailability of these piroxicam gel products.

2. *In Vivo* Evaluation of Percutaneous Absorption

The acidic nonsteroidal anti-inflammatory drugs (NSAIDs) have attained medical importance because of their ability to mitigate the symptoms of inflammation, particularly pain and swelling. NSAIDs are considered to have similar mechanisms of action, a major part of their activity being derived from inhibition of prostaglandin biosynthesis. This mechanism largely accounts for both their therapeutic activities and their common side effects. Thus the task that confronts the pharmacologist studying a particular NSAIDs is threefold: 1) to determine the extent to which the expected NSAIDs pattern of biological activities and side-effects is found in a particular compound, 2) to estimate the potency of these activities, and 3) to define the occurrence of biological activities, characteristics and properties unique to the particular chemical structure (Otterness and Bliven, 1985).

There are many models that are useful for pharmacological evaluation of the acidic NSAIDs. Some of these models are also of value for examining the activity of other classes of drugs useful for the treatment of inflammation. In addition, different NSAIDs exhibit different plasma half-life and metabolism and show different species and strain sensitivities. Different tests and variant methodologies further confound the simple

interpretation of data. As a result, animal tests provide only a probable constellations of effects which are rarely predicted in detail. Laboratory tests of NSAIDs activity may be expected to define the presence of such activity in animals and only provide an expectation for its occurrence in human and a guide for the execution of clinical trials (Otterness and Bliven, 1985).

There are a number of ways in which *in vivo* absorption can be assessed. Drug penetration is measured either by determining the amount of drug lost from the formulation, its appearance in the plasma, or its excretion in the urine and feces. Often, radiolabeled materials are employed, but it should always be realized that this will give concentrations which are the sum of the parent drug plus its metabolites. If the drug concentration alone is required, more specific assays should be employed.

A less quantitative means of assessing percutaneous absorption of NSAID is to observe their effect in reducing a previously induced inflamed symptom. This may be produced by, for example, UV irradiation, carrageenan-induced paw edema or adjuvant arthritis. Whatever the *in vivo* technique used for determining the formulation effects, an animal model will be more sensitive than that exhibited in humans (Hadgraft, 1989).

The purpose of this part of experiment was to compare different *in vivo* methods that have been used in the evaluation of percutaneous absorption of topical NSAIDs like piroxicam. When this drug was formulated into topical dosage forms, evaluation of their topical

bioavailability based on analysis of drug concentration in plasma usually posed a difficult task since the amount of the drug found in the blood is very low. Alternative *in vivo* techniques are thus available which are mostly based on the measurement of the NSAID pharmacological activities in animal models.

2.1 *In Vivo* Evaluation of Local Anti-Inflammatory Activities in Animal Models

Topical application of steroid is used for the treatment of various skin complaints such as eczema and contact dermatitis. The laboratory evaluation of the local anti-inflammatory activity of these compounds has involved models such as the granuloma pouch or ear irritation induced by croton oil (Dipasquale et al, 1970). There is a continuing search, however, for an additional model having a closer aetiological resemblance to human disease which might be even more predictive of a clinical response (Evan et al, 1971).

Inflammation is a sequence of response occurring in the body tissues initiated usually by noxious or injurious stimuli. Cellular and vascular alterations can be observed, and these are often quite similar whether the stimuli are chemical and physical in nature. Acute inflammation is characterized by swelling as a result of plasma and protein leakage from blood vessels into the extravascular (interstitial) space, and redness or erythema due to an increase in the local blood flow (hyperemia). It is obvious that inflammation is a complex series of *in vivo* events resulting from

an even more complex network of cell-cell, cell-mediator, and tissue interactions that cannot be reproduced in isolation *in vitro*. Therefore, while *in vitro* investigation facilitates dissection of specific events, the confirmation of any hypothesis based on *in vitro* data must be performed in the *in vivo* setting in which all the regulatory and counter regulatory, inflammatory, cellular and humoral mechanisms are operative and in which the physiological or pharmacological relevance of hypothesis can be evaluated (Issekutz and Issekutz, 1989).

Recent studies to produce models of allergic contact hypersensitivity have been successful, both from the standpoint of lesion induction and treatment. The animals are sensitized by painting the abdomen with the sensitizer (oxazolone or other). Several days later the sensitized animals are challenged on one ear by topical application of the same sensitizer. The contact delayed hypersensitization reaction can be quantified by measuring the swelling of the ear with micrometer. The mouse model has been used to investigate immunosuppressive properties of certain anti-inflammatory drugs (Bouclier et al, 1989).

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2.1.1 Measurement of Oxazolone-induced Ear Edema

The results of the *in vivo* evaluation of the four gel products for their anti-inflammatory activities using the oxazolone induced delayed hypersensitivity assay are shown in Table 8. It can be seen from the table that the greater the suppression of the ear edema, the smaller the difference in weight between the right and left ears. Thus, the most effective product

Table 8 : Comparison of oxazolone-induced ear weight difference of four piroxicam gel products and control group

Product	Ear weight difference* (mg)
A	10.05 ± 4.64
B	10.38 ± 4.83
C	9.16 ± 6.52
D	8.75 ± 4.67
Control	20.58 ± 3.70

* n = 12, mean ± SD

Table 9 : Percent suppressing ear edema of four piroxicam gel products

Product	**Percent suppressing ear edema
A	51.17
B	49.56
C	55.49
D	57.48

$$\text{**Percent suppressing ear edema} = \frac{\text{mean(Control)} - \text{mean(Product X)}}{\text{mean(Control)}} \times 100$$

should be able to decrease the weight of the edema-plagued right ear as close to the normal weight of the left ear as possible. In the control group no product was applied to the right ear following oxazolone sensitization. Therefore, the right ear became increasingly edematous with pronounced erythema at 24 hr post-challenge. As can be seen from Appendix VI, the weight of the right ear of each mouse in this group was much higher than the corresponding untreated left ear, leading to the largest weight difference (20.58 $\pm$ 3.70 mg).

From the results in this table, it is clearly seen that the four gel products containing piroxicam appeared to be effective in suppressing edema. The average weight differences were found to be in the range of 8.75 $\pm$ 4.67 to 10.38 $\pm$ 4.83 mg. By comparing these values to the control group, the percent reduction of edema was calculated to be 49.6 to 57.5% (Table 9).

2.1.1.1 Statistical Analysis of Ear Weight Difference

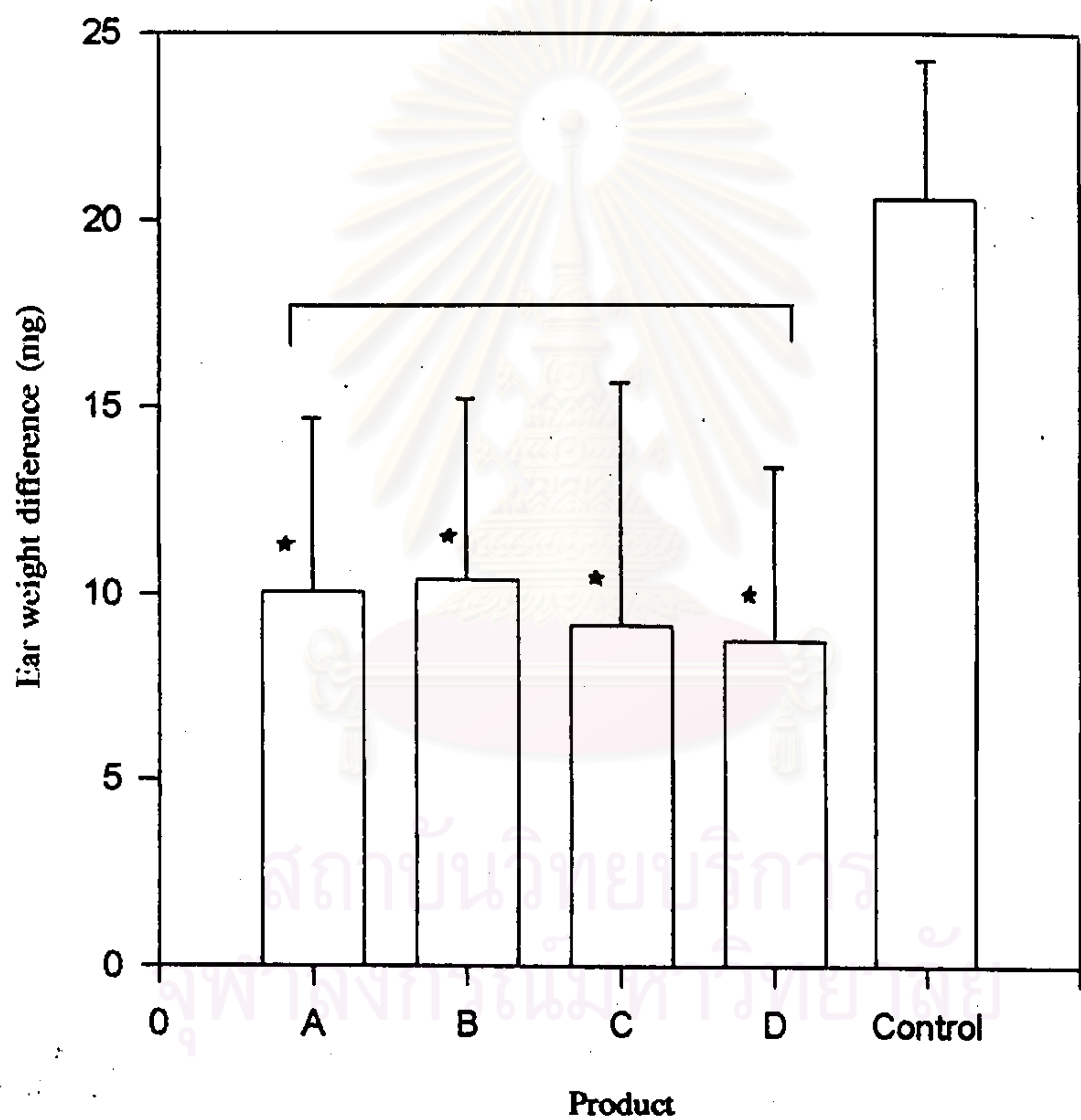
An analysis of variance (ANOVA) was applied to the data obtained from ear weight difference at 5% significance level (Appendix VII). A significant difference was found between the five groups ($F_{4,55}$ = 12.01, p = 0.0001) in the ear weight difference. The Duncan's multiple range test was further applied to the above data in an attempt to rank the effectiveness of each product at 5% significance level. The ranking result, in an increasing order, for the ability to suppress the oxazolone-induced ear edema in mice is Control $< \underline{B < A < C < D}$. Results from the Duncan's test supported the above findings that the line underneath the letter A, B, C and D signify that

there was no significant difference between the four products ($p > 0.05$). However, the products A, B, C, D were all equally effective in reducing ear edema as compared to the control group ($p < 0.05$). The results are also graphically represented in Figure 21.

Although the skin of human being is different from that of the common laboratory animals and hence the ease with which drugs will penetrate to the deeper layers might be different among different species, useful information can still be obtained from the animal experiments. There is a need for a technique suitable for the assessment of the effects of anti-inflammatory agents after topical application which is simple and reliable. The induction of contact sensitivity in the mouse using oxazolone is a straightforward procedure and the increase in the size of the treated ear over the normal is of the order of 100%. The technique gave reproducible results and was quantitative (Evans et al, 1971).

In this experiment, it was found that the assessment of anti-inflammatory activity of piroxicam showed a good result, with all gel products being significantly effective in reducing ear edema as compared to the control group. However, as can be seen from the results, the four products were also equally effective in reducing edema. Apparently, this technique may not be sensitive enough to distinguish the topical bioavailabilities of piroxicam gel products with widely different *in vitro* release/permeation characteristics.

Figure 21 : Comparison of the ear weight difference from oxazolone-induced ear edema of four piroxicam gel products and control group (Value = mean ± SD, n = 12)



* Significantly different from control using Duncan's test at 5% level The line joining products A, B, C and D indicates that the four products are equally effective in reducing ear edema.

Oxazolone, a product of efforts to synthesize penicillin in the 1940s, is an extremely potent contact-sensitizing agent for human and rodent. In rodents, the induction of a sensitized state to oxazolone occurs maximally within 5 days of the primary topical application of a 1-2% oxazolone in acetone solution. The response to a second (challenge) dose of oxazolone to the ear begins with the appearance of erythema within 4 hr, followed by ear edema, which reaches a maximum between 6 and 8 hr and remains intense through at least 96 hr. The response to a challenge to the mouse ear, as measured by ear edema 24 hr later, is most intense when the challenge occurs 5-10 days after the sensitizing dose. Histologically, the challenge reaction proceeds through intense vasodilatation and edema beginning at 4 hr, and the extravasation of lymphocytes, macrophages and granulocytes at 6 hr. By 24 hr postchallenge, the dermis become spongiotic and uniformly infiltrated with mononuclear cells and granulocytes; in some area the epidermis is detached or necrotic. At 48 hr, mononuclear cells predominate, and epidermal necrosis is frequently extensive. The healing period beyond 5 days consists of epidermal regeneration and a gradual reduction in the number of leukocytes present in the dermis. In the absence of effective drug treatment, substantial portions of full-thickness ear tissue may be lost to necrosis. Studies by Griswold indicated that there action is primarily cell-mediated; naive mice (no previous contact with oxazolone) can be rendered sensitive by the transfer of cell, lymphocytes or splenocytes, obtained from sensitized animals (Young and De Young, 1989)

Also, the use of chemical sensitizing agents does involve some hazard to the experimentor due to the risk of skin sensitization following, for example, accidental spillage. Precautions, including the wearing of gloves and safety glasses and the rinsing of contaminated glassware with diluted ammonia, which degrades the compound to non-sensitizing products, must be adopted (Evans et al, 1971).

2.1.2 Measurement of Vascular Permeability Changes

After the products were applied to the rat dorsal skin. The permeability response was induced by injecting histamine intradermally into the center of the treated site of the rat skin. Histamine rapidly increases the intensity of the blue dye spot after an intravenous injection of pontamine sky blue. Twenty minutes after the individual rats were given an intradermal injection of histamine and intravascular injection of pontamine sky blue, animals were decapitated and the treated skin removed. The result of four gel products on their inhibition of histamine-induced vascular permeability are shown in Table 10. It was found that the smaller the size of blue spots, which were obtained by multiplying the shortest by the longest diameter and expressed in mm^2, the greater the inhibition of permeability by the individual products. When compared with the control group (no product was applied to the skin), it can be seen that the average sizes of the blue spots of the rats in the drug treated groups were smaller than the control group (246.19 ± 28.08 mm^2).

Table 10 : Comparison of the blue spot size of four piroxicam gel products and control group, induced by histamine

Product	Size of blue spot* (mm^2)
A	212.12 ± 31.97
B	200.02 ± 19.82
C	230.48 ± 20.41
D	205.04 ± 27.17
Control	246.19 ± 28.08

*n = 10, mean ± SD

Table 11 : Percent inhibition of vascular permeability of four piroxicam gel products

Product	**Percent inhibition of vascular permeability
A	13.84
B	18.75
C	6.38
D	16.71

**Percent inhibition of vascular permeability

$$= \frac{\text{mean(Control)} - \text{mean(Product X)}}{\text{mean(Control)}} \times 100$$

From this table, the four piroxicam gel products appeared to be quite effective in decreasing the vascular permeability induced by histamine, with the average size of the blue spots ranging from 200.02 ± 19.82 to 230.48 ± 20.41 mm^2.

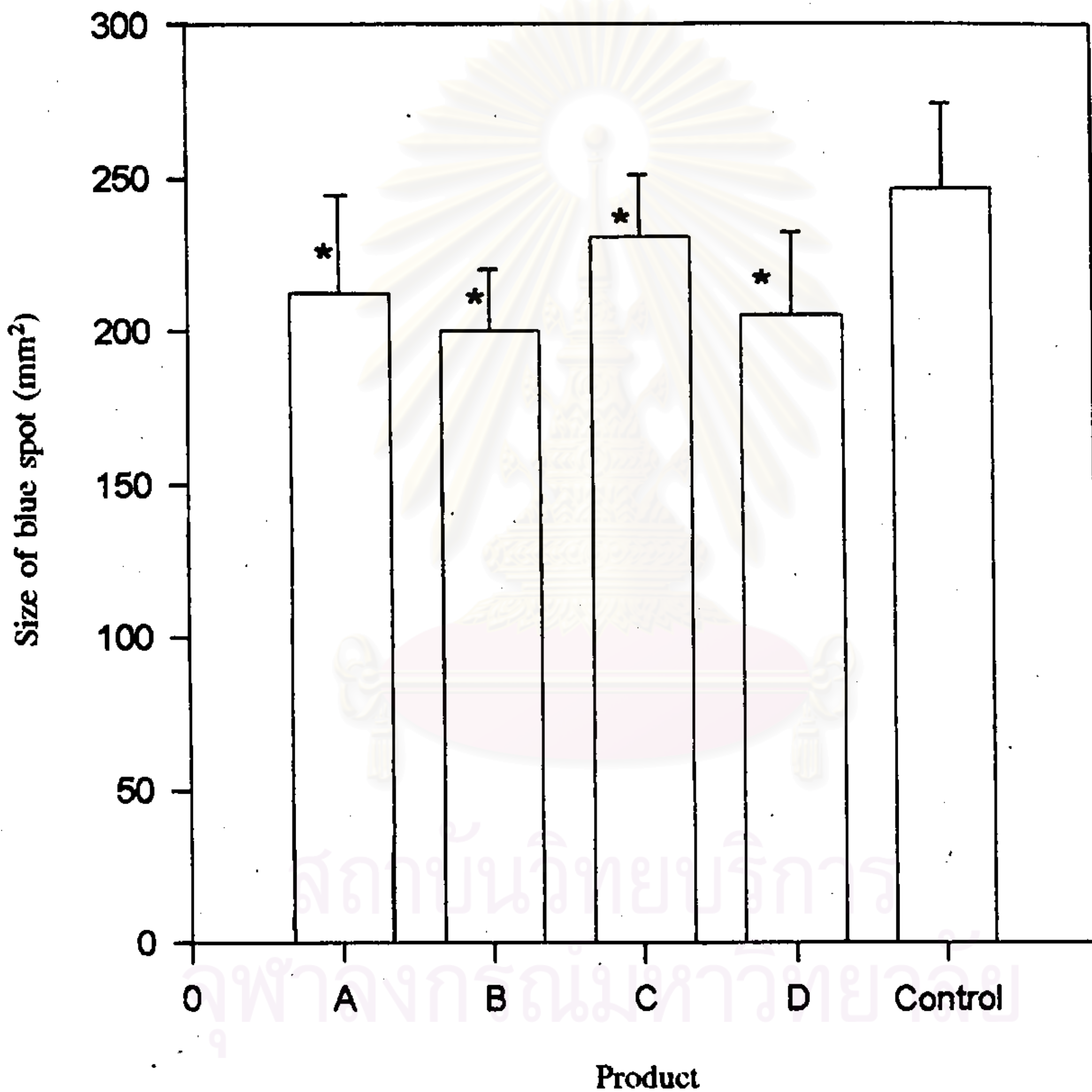
By comparing to the control group, the percent inhibition of vascular permeability was calculated to be 6.38 to 18.75% as shown in Table 11.

2.1.2.1 Statistical Analysis of Inhibition of Vascular Permeability

An analysis of variance (ANOVA) was applied to the data obtained from the sizes of the blue spots at 5% significance level (Appendix VII). A significant difference was found between the five groups ($F_{4,45} = 5.01$, $p = 0.0024$) in the size of the blue spots. Comparison of the average sizes of blue spots is shown in Figure 22.

The Duncan's multiple range test was further applied to these data in an attempt to rank the effectiveness of each product at 5% significance level. The ranking result in an increasing order, for inhibition of vascular permeability in rat is Control < C < A < D < B. However it is difficult to differentiate in detail the relative efficacy of each product because there were three lines underneath the letters. According to Duncan's test, product C was not different from the control but products A, D and B were all effective ($p < 0.05$) over the control. In addition, products C, A and D also form another

Figure 22 : Comparison of the blue spot size of four piroxicam gel products and control group (Value = mean ± SD, n = 10)



* Significant when compared to control group using Dunnett's test at 5%level

group having an intermediate effect between control and product B. The Duncan's test result apparently made it quite complicate to distinguish among these products. Therefore, another post-ANOVA test, namely the Dunnett's test was applied to compare the efficacy of each product relative to only the control group. It can be concluded that products A, B, C and D showed a statistically significant effect in the inhibition of vascular permeability over the control group ($p < 0.05$). The result are also graphically represented in Figure 22.

In the inflammatory response to injury, increased capillary permeability is an early and important vascular event. Research into the mediation of the permeability changes generally concerns endogenous substances with high permeability increasing potency like histamine. These substances are likely to be the natural mediators in inflammation, particularly if they are widely distributed in mammalian tissues and are readily activated. Various endogenous permeability factors satisfying these criteria have been identified; and, in most cases, detected in inflammatory exudates. However, their presence in exudates is insufficient to establish their relevance as the natural mediators of the appropriate permeability changes. Established candidates for this role can be divided into two groups. The first group consists of amines, histamine and 5-hydroxytryptamine, and the latter group consists of proteases and their products of proteolysis. Permeability changes are readily investigated in the skin of animal with a vital dye like pontamine blue circulating in their blood since the increased permeability to large

molecules would lead to local exudation of the blue dye at a treated site (Wilhelm & Mason, 1960).

The immediate response in the guinea-pig and rabbit is mediated by histamine. Small amounts of free histamine sufficient to induce the observed response are recoverable exudation of dye and observed edema, in both species is abolished by low concentrations of local or systemic antihistamine. The local edema that appears in the rat is only moderately suppressed by antihistamine (Logan and Wilhelm, 1960).

Chemical injury has long been a popular experimental model for studies of the inflammatory response, particulary for the identification of the characteristic vascular reactions. One of the best known studies is that of Lewis and his colleagues who noted that, when pricked into the skin, acids and alkalies evoked an immediate "triple response" similar to that with histamine. The early triple response appeared to be induced by histamine or related "H-substance", whereas the slower response with substance like chloroform (Steele and Wilhelm, 1966).

Williams and Jose (1981) indicate that a substance is generated in the skin which acts synergistically with endogenous, or exogenous, vasodilator protaglandin to produce inflammatory edema. Because both histamine and bradykinin are potent vascular permeability-increasing substances whose effects can be similarly enhanced by protaglandin.

Changes in vascular permeability and potential antiprokiferative effects were determined to assess activity against secondary effects of inflammation. The biological activities of topically applied anti-inflammatory agents have been assessed in animal models of inflammation to provide supportive evidence of their effective transcutaneous absorption. Although animal skin is more permeable than human skin, studies of the efficacy of drugs in animal model can provide valuable information: an agent showing litter or no activity in animals is unlikely to possess any effect in man (Hiramatsu et al, 1990).

The appropriate use of animal models for the study of NSAIDs requires detailed examination of the models from several perspectives. First, the experimental details of the model need to be clearly understood about the operation of the model and the determined biological effects. Often, experimental or procedural differences between laboratories are sufficiently large that results cannot be readily compared. While these differences may not invalidate a model, they do suggest that experimental procedures must be carefully examined so that trivial and non-meaningful results can be eliminated and the operating procedure most relevant to human therapeutics can be utilized. Second, the operand mechanisms of the model, and a correlative question, the importance of these mechanisms in human disease, need to be understood in order to evaluate the limitations of extrapolation from the animal model to human disease. Finally, when useful drug therapy already exists, the correlation between predictions of effectiveness in the clinic can be subjected to detailed examination. While any correlation

between animal model and clinic must be approximate, it serve as a useful guide to whether continued testing in the model is likely to result in the desired type of therapeutic agent. However, good NSAIDs activity coupled with an improved activity profile in animal models enhances the odds that biologically and structurally novel NSAIDs will be tested clinically, and therefore improves the odds of discovering in human an advantageous therapeutic activity (Otterness and Bliven et al, 1985).

2.2 Application of *In Vivo* Skin Stripping Technique to Evaluate Percutaneous Absorption of Piroxicam Gel Products

It was very interesting to find an alternative *in vivo* technique which was able to distinguish, quantitatively, the topical bioavailability of various piroxicam gel products.

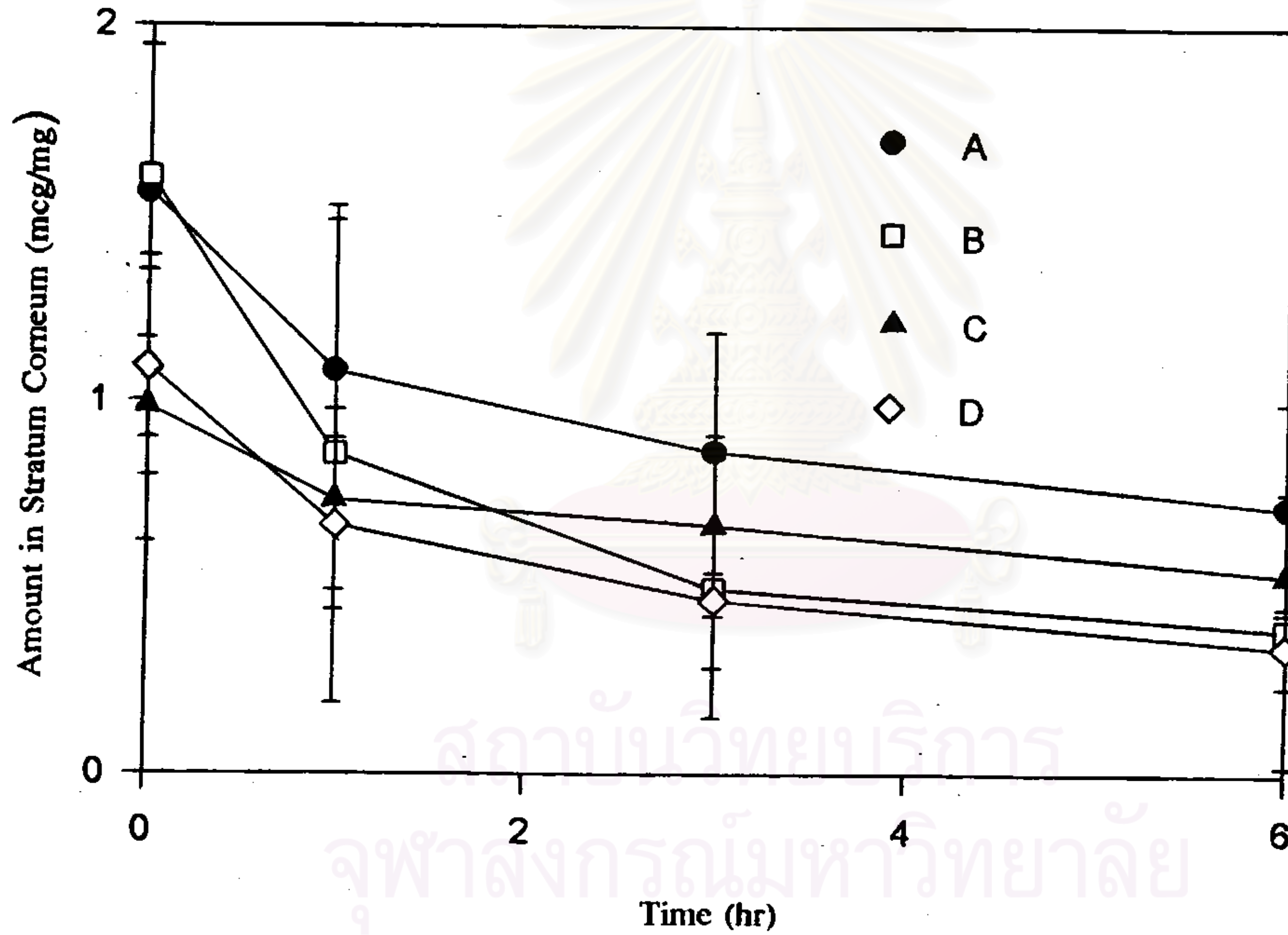
Following Samitamarn's study to ensure the suitability of the system, eight healthy volunteers (four males and four females, age ranging from 22-30 years) participated in this study. The study was a crossover design in which each subject received all the four products (A, B, C, D,) but on different occasions separated by two week washout period. The treatment sequence was completely randomized for each subject.

Each product was topically applied to the left forearm of the individual subjects. Then the treated area was occluded for 3 hr to allow for the maximum release and penetration of the drug into the stratum corneum (Samitamarn, 1995). The excess gel was then removed from each of the

application spots with the use of cotton buds to terminate drug administration. As described in Chapter III , there were a total of twelve application spots in the forearm of each subject (four rows of three spots each; Figure 13 of Chapter III). Immediately following removal of excess gel, one row of the application spots was randomly selected for stripping to determine the initial amount of piroxicam in the stratum corneum, i.e. the amount at time zero. The tapes were weighed before and after skin stripping in order to obtain the weight of the removed stratum corneum. Another row was then randomly chosen for stripping at 1 hr after termination of drug treatment to determine the amount remaining at 1 hr. Previous experiments have shown that there was no significant difference with the respect to the amount of drug found in the stratum corneum among the closely parallel rows within the same forearm after the same period of occlusion and/or skin stripping (Tengamnuay et al, 1996). Consequently, the other two rows were randomly stripped at 3 and 6 hr, respectively, to determine the amount of piroxicam remaining in the stratum corneum at 3 and 6 hr. Loss of piroxicam from this layer upon termination of drug application would imply the occurrence of percutaneous absorption to the deeper skin layers and underlying tissues.

The amount of piroxicam found in the stratum corneum removed from each subject at time 0, 1, 3 and 6 hr following termination of drug application are shown in Tables 12-15 for the four products evaluated. The plots of the amount as a function of time are provided in Figure 23 for the four products (average of 8 subjects). The HPLC data, the weight of the stratum corneum and plots of the amount of drug remaining in the stratum

Figure 23: Absorption profiles of four piroxicam gel products through stratum corneum (Value = mean ± SD, n = 3)



corneum versus time for individual subjects are also given in Appendix VIII. From these figures, it can be seen that there was a gradual decrease with time in the piroxicam amount in the stratum corneum, in all subjects and products evaluated. This indicated that following the termination of drug administration, piroxicam was substantially absorbed from the stratum corneum into the deeper skin layers. In addition, due to the previous removal of the excess gel before analyzing the amount of drug in the stratum corneum as a function of time, the process of drug release from the vehicle was thus eliminated from the overall percutaneous absorption process. Penetration of piroxicam into the deeper skin layers was the only process responsible for the loss of drug from the stratum corneum. Therefore, it should be possible to calculate the extent of percutaneous absorption using the following equation ;

$$\text{\% piroxicam percutaneously absorbed} = \frac{(\text{amount at time zero} - \text{amount at time t}) \times 100}{\text{amount at time zero}}$$

On the other hand, the processes of drug release and penetration were occurring simultaneously during the 3 hr occlusion period. The two components both contributed to the overall percutaneous absorption. As a result, it was difficult to determine the percent drug actually absorbed during this period.

The amount of piroxicam in stratum corneum found at time zero was the highest with product B, the average value being 1.600 ± 0.700 μg/mg

(Table 13). Product A gave the second highest value of 1.557 ± 0.389 μg/mg (Table 12), followed by product D at 1.091 ± 0.294 μg/mg (Table 15). Product C gave the lowest ratio at 0.986 ± 0.364 μg/mg (Table 14). Since these values were the amounts that were found in the stratum corneum immediately after removal of the excess gel, it was possible that the differences observed among the four products at this time point may have been due to the differences in the drug release and/or penetration rates during the 3 hr occlusion period. Table 16 summarizes the amount remaining in the stratum corneum removed at various times for the four products.

Randomized block ANOVA was applied to test for any significant differences in the amounts of piroxicam found at time zero among the four products at 5% level, the statistical results are given in Appendix VIII, the data indicated that there was a significant difference among the four products with respect to the ratio of the initial amount in the stratum corneum ($F_{3,21}$ = 3.5062, $p = 0.0328$). Duncan's multiple range test was further applied to rank the observed difference at 5% level. The results are also shown in Appendix VIII and summarized in Table 17. From this table, the ranking of the initial amount of piroxicam in the stratum corneum found in the tape-stripped stratum corneum, in an increasing order, was C < D < A < B. The same notation was applied in the data interpretation, i.e. the lines underneath the letters C and D and the letters D, A and B indicated that there was no significant difference between the products above each line ($p > 0.05$).

Table 12 : The amount of piroxicam in stratum corneum at various time

(The individual value is an average of pooled 3 spots in the same row)

Product A :

Subject No.	Amount in stratum corneum (μg/mg) at			
	0 hr	1 hr	3 hr	6 hr
1	0.934	0.907	0.858	0.801
2	1.363	0.653	0.339	0.319
3	1.541	0.963	0.890	0.705
4	2.069	1.887	1.519	1.258
5	2.013	1.558	1.079	1.003
6	1.878	1.126	0.851	0.484
7	1.558	0.851	0.749	0.618
8	1.098	0.703	0.619	0.551
mean ± SD	1.557 ± 0.389	1.081 ± 0.403	0.863 ± 0.321	0.717 ± 0.281

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Table 13 : The amount of piroxicam in stratum corneum at various time

(The individual value is an average of pooled 3 spots in the same row)

Product B :

Subject No.	Amount in stratum corneum (μg/mg) at			
	0 hr	1 hr	3 hr	6 hr
1	2.529	2.477	1.339	1.310
2	1.380	1.142	0.499	0.251
3	0.858	0.339	0.256	0.213
4	0.968	0.257	0.130	0.111
5	2.372	0.482	0.278	0.218
6	2.426	0.777	0.560	0.482
7	1.056	0.688	0.418	0.126
8	1.207	0.696	0.494	0.411
mean ± SD	1.600 ± 0.700	0.857 ± 0.665	0.497 ± 0.346	0.390 ± 0.368

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Table 14 :The amount of piroxicam in stratum corneum at various time

(The individual value is an average of pooled 3 spots in the same row)

Product C :

Subject No.	Amount in stratum corneum (μg/mg) at			
	0 hr	1 hr	3 hr	6 hr
1	1.648	1.055	0.948	0.540
2	0.706	0.602	0.525	0.439
3	0.550	0.450	0.439	0.223
4	0.535	0.375	0.311	0.436
5	1.127	0.976	0.877	0.794
6	1.346	0.612	0.447	0.357
7	0.968	0.896	0.884	0.813
8	1.006	0.924	0.893	0.682
mean ± SD	0.986 ± 0.364	0.736 ± 0.241	0.666 ± 0.242	0.536 ± 0.198

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Table 15 :The amount of piroxicam in stratum corneum at various time

(The individual value is an average of pooled 3 spots in the same row)

Product D :

Subject No.	Amount in stratum corneum (μg/mg) at			
	0 hr	1 hr	3 hr	6 hr
1	0.924	0.494	0.350	0.307
2	1.583	0.462	0.334	0.298
3	0.965	0.605	0.425	0.387
4	0.733	0.610	0.440	0.299
5	0.719	0.503	0.270	0.173
6	1.132	0.829	0.594	0.318
7	1.421	1.200	0.884	0.575
8	1.253	0.661	0.442	0.378
mean ± SD	1.091 ±0.294	0.670 ± 0.228	0.467 ±0.181	0.342 ±0.107

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Table 16 : The amount of piroxicam in stratum corneum of four gel products at various time (Each value = average of 8 subjects)

Products	*Amount in stratum corneum (μg/mg)			
	0 hr	1 hr	3 hr	6 hr
A	1.56 ± 0.39	1.08 ± 0.40	0.86 ± 0.32	0.72 ± 0.28
B	1.60 ± 0.70	0.86 ± 0.66	0.50 ± 0.35	0.39 ± 0.37
C	0.99 ± 0.36	0.74 ± 0.24	0.67 ± 0.24	0.54 ± 0.20
D	1.09 ± 0.29	0.67 ± 0.23	0.47 ± 0.18	0.34 ± 0.10

*n = 8, mean ± SD

Table 17 : Comparison of the amount of piroxicam in stratum corneum of four gel products at various time

Time (hr)	F-Value	P-Value	Duncan's test (at $\alpha = 0.05$)
0	3.5062	p = 0.0328*	C < D < A < B
1	1.1622	p = 0.3480	D < C < B < A
3	2.7919	p = 0.0648	D < B < C < A
6	2.8713	p = 0.0599	D < B < C < A

* = Significant at 0.05

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Randomized block ANOVA was also applied to the data at 1, 3, and 6 hr. The results are given in Appendix VIII. As can be seen from these data, there was no significant difference among the four products with respect to the amount of piroxicam in the stratum corneum at either 1, 3 and 6 hr ($p > 0.05$). The Duncan's test also gave the same results as shown in Table 17. From this table, the ranking at 1 hr (D < C < B < A), 3 and 6 hr (D < B < C < A) were found to be different from the result at time zero (C < D < A < B). It appeared that the four products showed equivalent amount of drug remaining in the stratum corneum at 1, 3 and 6 hr as opposed to significant difference observed at time zero. However, it can be seen that the absorption of drug at 6 hr were slowly when compared with the absorption at 1 and 3 hr, the concentration of drug in the skin were almost saturated when closely 6 hr, as shown in Figure 23. The reason for such observed differences was not clearly known, so this result may effect to the statistical tests.

The percent of piroxicam percutaneously absorbed after termination of drug treatment was calculated for each product for each subject at time 1, 3 and 6 hr, according to the above equation. The data are summarized in Table 18 whereas the individual data are provided in Appendix IX. The results revealed that the percent drug absorbed during these periods was rather different between the four products. At 1 hr after termination of treatment, the percent loss of drug from the stratum corneum (which implied percutaneous absorption) was in the range of 22.79 to 45.04 % for the four products. After 3 hr more piroxicam had permeated through this layer and

Table 18 : Percent absorbed of piroxicam of four gel products at various time

Products	% Absorbed of piroxicam*		
	1 hr	3 hr	6 hr
A	30.66 ± 16.44	43.59 ± 18.52	52.36 ± 18.61
B	45.04 ± 28.16	71.72 ± 13.52	78.10 ± 14.20
C	22.79 ± 15.23	29.88 ± 18.14	41.78 ± 20.70
D	36.38 ± 17.17	57.28 ± 13.77	68.03 ± 7.67

*n = 8, mean ± SD

Table 19 : Comparison of percent absorbed of piroxicam in four gel products at various time

Time (hr)	F-Value	P-Value	Duncan's test (at $\alpha = 0.05$)
1	1.4092	p = 0.2674	C < A < D < B
3	8.3051	p = 0.0011*	C < A < D < B
6	7.1798	p = 0.0020*	C < A < D < B

* = Significant at 0.05

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the percent penetration of the four products was in the range of 29.88 to 71.72%. At 6 hr there was 41.78 to 78.10 percent of piroxicam penetration.

Randomized block ANOVA was then applied to this set of data at 5% level. The statistical results are given in Appendix IX and also summarized in Table 19. From this table, it can be seen that there was no significant differences among the four products with respect to the percent absorbed at 1 hr ($p > 0.05$). At 3 and 6 hr the percent absorbed was found to be significantly different as can be seen from the p-value which were far below 0.05 ($p = 0.0011$ and 0.0020). This implied that, at the same time point, piroxicam was percutaneously absorbed from the four products at different rates. Such an implication also helped explain the differences in the ranking result of the four products with respect to the amount of piroxicam in the stratum corneum at time 0, 1, 3 and 6 hr (Table 17).

The apparent first order rate constants for the percutaneous absorption process of the four piroxicam gel products were then calculated from the plot of the natural log of the amount in the stratum corneum at various times. According to the first order diffusion-controlled skin permeation, the plots should be linear and the slopes of such plots should represent the first order rate constants of percutaneous absorption. The numerical data of the rate constant values are given in Table 20. As can be seen from these figures, such plots give relatively linear relationships with regression coefficients (r^2) in the range of 0.8489 to 0.8994 within 6 hr (Appendix X). The linearity of these first order plots, as judged from the r^2

Table 20 : Rate constants of four piroxicam gel products over 6 hr

Product	Rate constants* (μg/mg.hr)
A	0.1234 ± 0.0604
B	0.2490 ± 0.0792
C	0.1093 ± 0.0864
D	0.1805 ± 0.0351

*n = 8, mean ± SD

Table 21 : Comparison of the rate constants of the four piroxicam gel products over 6 hr

	F-Value	P-Value	Duncan's test (at $\alpha = 0.05$)
Rate constants	5.8378	p = 0.0049*	C < A < D < B

* = Significant at 0.05

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values, apparently was not remarkable. This could be partly explained by the relatively slow drop of the piroxicam content in the stratum corneum at 6 hr. As a result, calculation of the first order rate constants over the 6 hr period is only approximate and may not reflect a simple process of drug diffusion through various skin layers.

Randomized block ANOVA was then applied to these data at 5% level. The statistical results are given in Appendix X and also summarized in Table 21. From this table, it can be seen that there were significant differences among the four gel products with respect to the first order percutaneous absorption rate constants. This indicate that the loss of drug from the stratum corneum appeared not to follow the same kinetics among the four products. Differences in percutaneous absorption kinetics may therefore account for the deviation from the simple first order process. This would, in turn, make it unrelevant to directly compare the extent of percutaneous absorption at various times among these products since their absorption kinetics were not the same. In this case, the percent absorbed at different times may better represent the topical bioavailability, particularly with respect to the rate of absorption.

3. Correlation Studies between *In Vitro* Release/Permeation and *In Vivo* skin Stripping Data

Following an initial observation of the data from the *in vitro* and *in vivo* studies, there appeared to be different in the ranking results of piroxicam diffusion through either cellulose acetate or new born pig skin membrane and

the amount of piroxicam in the stratum corneum at all time points. For example, the *in vitro* release rate through the cellulose acetate membrane gave the ranking of the four products, in an increasing order, to be $\underline{A \leq D} < C < B$ whereas the permeation flux through the new born pig skin was $\underline{A \leq C \leq B} < D$. On the other hand, the results from Table 17 indicated that the ranking order of the piroxicam content in the stratum corneum at time zero was $\underline{C \leq D < A} < B$. When test for correlation was applied at 5% level, no correlation was found between the *in vitro* release/permeation parameters and the *in vivo* initial content of piroxicam in the skin since the p-value was greater than 0.05 in all cases (Table 22). Actually, the results from Table 22 indicated that there was no correlation whatever between the *in vitro* parameters (release rate, permeation flux, cumulative amount released, and cumulative amount permeated) and the *in vivo* skin stripping parameters (piroxicam content in the stratum corneum at time 0, 1, 3 and 6 hr, apparent first order rate constant, percent absorbed at 1, 3 and 6 hr, and area under the piroxicam content in the stratum corneum versus time curve ($AUC_{0\text{-}6hr}$); data in Appendix XI). In addition, there were no correlations between the *in vitro* release/permeation parameters and the *in vivo* pharmacological response parameters (i.e. ear weight difference and size of the blue spots) as observed from the results in Table 22 and data in Appendix XII. Since the results from the *in vitro* experiments are usually employed to predict the *in vivo* performance of the products provided that significant correlations can be established, lack of the *in vitro-in vivo* correlations observed here thus indicate that the *in vitro* release/permeation models described in this study

Table 22 : Correlation test of *in vitro* release/permeation and *in vivo* skin stripping/pharmacological studies

In Vivo	***In Vitro*** **release/permeation studies**		**Correlation coefficient (r)**	**P-Value**
Amount in St. corneum at 0 hour	Cellulose acetate membrane	Release rate	0.2693	$p > 0.05$
		Cumulative amount	0.2114	$p > 0.05$
	New born pig skin	Steady state flux	0.5044	$p > 0.05$
		Cumulative amount	0.4912	$p > 0.05$
Amount in St. corneum at 1 hour	Cellulose acetate membrane	Release rate	0.1817	$p > 0.05$
		Cumulative amount	0.2029	$p > 0.05$
	New born pig skin	Steady state flux	0.7217	$p > 0.05$
		Cumulative amount	0.7250	$p > 0.05$
Amount in St. corneum at 3 hour	Cellulose acetate membrane	Release rate	0.5123	$p > 0.05$
		Cumulative amount	0.4829	$p > 0.05$
	New born pig skin	Steady state flux	0.6881	$p > 0.05$
		Cumulative amount	0.7062	$p > 0.05$

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Table 22 : Correlation test of *in vitro* release/permeation and *in vivo* skin stripping/pharmacological studies (continue)

In Vivo	***In Vitro* release/permeation studies**		**Correlation coefficient (r)**	**P-Value**
Amount in St. corneum at 6 hour	Cellulose acetate membrane	Release rate	0.4291	$p > 0.05$
		Cumulative amount	0.3992	$p > 0.05$
	New born pig skin	Steady state flux	0.7534	$p > 0.05$
		Cumulative amount	0.7696	$p > 0.05$
Rate Constants over 6 hour	Cellulose acetate membrane	Release rate	0.6940	$p > 0.05$
		Cumulative amount	0.6295	$p > 0.05$
	New born pig skin	Steady state flux	0.2384	$p > 0.05$
		Cumulative amount	0.2646	$p > 0.05$
% Absorbed at 1 hour	Cellulose acetate membrane	Release rate	0.5242	$p > 0.05$
		Cumulative amount	0.4464	$p > 0.05$
	New born pig skin	Steady state flux	0.2346	$p > 0.05$
		Cumulative amount	0.2584	$p > 0.05$

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Table 22 : Correlation test of *in vitro* release/permeation and *in vivo* skin stripping/pharmacological studies (continue)

In Vivo	***In Vitro* release/permeation studies**		**Correlation coefficient (r)**	**P-Value**
% Absorbed at 3 hour	Cellulose acetate membrane	Release rate	0.5102	$p > 0.05$
		Cumulative amount	0.4323	$p > 0.05$
	New born pig skin	Steady state flux	0.2940	$p > 0.05$
		Cumulative amount	0.3177	$p > 0.05$
% Absorbed at 6 hour	Cellulose acetate membrane	Release rate	0.4862	$p > 0.05$
		Cumulative amount	0.4091	$p > 0.05$
	New born pig skin	Steady state flux	0.3789	$p > 0.05$
		Cumulative amount	0.4020	$p > 0.05$
AUC $_{(0\text{-}6\ hr)}$	Cellulose acetate membrane	Release rate	0.3185	$p > 0.05$
		Cumulative amount	0.3122	$p > 0.05$
	New born pig skin	Steady state flux	0.7863	$p > 0.05$
		Cumulative amount	0.7968	$p > 0.05$

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Table 22 : Correlation test of *in vitro* release/permeation and *in vivo* skin stripping/pharmacological studies (continue)

In Vivo	***In Vitro* release/permeation studies**		**Correlation coefficient (r)**	**P-Value**
Ear weight difference	Cellulose acetate membrane	Release rate	0.4845	$p > 0.05$
		Cumulative amount	0.4520	$p > 0.05$
	New born pig skin	Steady state flux	0.7376	$p > 0.05$
		Cumulative amount	0.7230	$p > 0.05$
Size of blue spot	Cellulose acetate membrane	Release rate	0.2307	$p > 0.05$
		Cumulative amount	0.1429	$p > 0.05$
	New born pig skin	Steady state flux	0.3424	$p > 0.05$
		Cumulative amount	0.3606	$p > 0.05$

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appear to have limited predictive value and may not represent reliable *in vitro* models for evaluation of topical piroxicam gel products.

The lack of correlation between the *in vitro* release through the cellulose acetate membrane and the *in vivo* skin stripping parameters with piroxicam gels was in contrast to the results of diclofenac diethylammonium gels as reported by Samitamarn (1995). Using the same experimental procedures, she found that there was significant correlation between the *in vitro* release of diclofenac from the gel base and the amount of diclofenac detected in the stratum corneum at time 0, 1 and 3 hr as well as $AUC_{0\text{-}6\ hr}$. The disprecancies in the results between piroxicam and diclofenac could be partly explained by differences in the physiochemical properties of the two drugs themselves. The other reasons could be due to differences in the kinetics of percutaneous absorption between the two drugs. Diclofenac was found to be percutaneously absorbed through the stratum corneum of the human forearm by an apparent first order diffusion process. Semilogarithmic plots of the amount of diclofenac remaining in the stratum corneum as a function of time gave straight lines for all the four gel products evaluated, with relative good regression coefficient. The data for average apparent first order rate constants for the loss of diclofenac from the stratum corneum are given in Table 23 for comparison with those of piroxicam obtained in this study. The most interesting result observed with diclofenac was that apparent first order rate constants of the four gel products were not statistically different among each other ($p > 0.05$) (Table 23). This implied that diclofenac appeared to be percutaneously absorbed through the stratum

Table 23 : Rate constants of four diclofenac gel products over 6 hr and their Duncan's test results (Samitamarn, 1995)

Product	Rate constants* (μg/mg.hr)
A	0.3989 ± 0.1502
B	0.3335 ± 0.0804
C	0.2676 ± 0.1266
D	0.3920 ± 0.0759
F-Value	1.9270
P-Value	p = 0.1553*
Duncan's test (at α = 0.05)	C < B < D < A (no significant difference)

*n = 8, mean ± SD

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corneum with the same first order absorption kinetics. This would also lead to non-significant differences observed with the percent absorbed at 0, 1 and 3 hr since the rate constants (k) of the four products were similar. As shown in Table 24 ANOVA at 5% level showed that the percent absorbed of diclofenac at these time points were not significantly different among the four products tested. Furthermore, by comparing the percent *in vitro* release of diclofenac at 6 hr with the percent percutaneous absorbed after skin stripping, data in Table 26 clearly indicated that diclofenac was released at a rate which was much slower than the penetration rate through the human stratum corneum in all the four products. For example, at the same 6 hr period, only about 8-12 percent of diclofenac was released from the four products whereas about 78-90 percent has already been absorbed by this time for all the four products. This finding suggested that the release of diclofenac from the vehicle appeared to be the rate-limiting step in the overall percutaneous absorption of this drug and also helped explain the significant correlations observed between the *in vitro* release and the *in vivo* skin stripping parameters (Tengamnuay and Samitamarn, 1996). As previously stated, the amount of the drug found in the stratum corneum immediately after 3 hr-occlusion (at time zero) was a result of two simultaneous processes, i.e. the release of the drug from the gel base onto the stratum corneum surface and the penetration of the drug from through this skin layer. Since the penetration kinetics was the same for four products and the release of the drug occurred at a rate much slower than the absorption, the difference in the amount of drug detected in the stratum corneum at time zero was mainly a result of the difference in the release characteristics among the four products. The ranking

Table 24 Average percent of diclofenac percutaneously absorbed from four diclofenac gel products and their ANOVA test results (Samitamarn, 1995)

***n = 8, mean ± SD**

Time (hr)	% Absorbed of diclofenac *				ANOVA Results
	A	B	C	D	
1	63.06 ± 13.40	58.85 ± 21.74	56.26 ± 22.16	61.95 ± 23.80	$p > 0.05^{NS}$
3	79.39 ± 9.85	77.50 ± 17.93	71.66 ± 26.30	84.91 ± 10.96	$p > 0.05^{NS}$
6	89.72 ± 7.82	87.12 ± 13.50	78.50 ± 20.83	85.94 ± 15.89	P = 0.045

NS = not significant

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of the four products with respect to the amount initially detected in the stratum corneum exactly matched that of the *in vitro* release rate. In addition, the same ranking of diclofenac content at time zero also persisted to later time points (1, 3 and 6 hr) as a result of the same kinetics of percutaneous absorption when the process of drug release has been eliminated by wiping off the excess gel at the end of the occlusion period.

On the contrary, results with piroxicam gels obtained in this study were different from diclofenac. Although the penetration process appeared to involve diffusion/partition of the drug through various skin layers as suggested by the linearity of the first order plots, the apparent first order rate constants for penetration of piroxicam through the stratum corneum of human forearm were found to be statistically different among the four products ($p < 0.05$, Tables 20 and 21) as opposed to diclofenac results. Differences in the rate constants implied that the four piroxicam products were absorbed at different first order kinetics. This has also led to significant differences in the percent piroxicam absorbed at 1, 3 and 6 hr (Tables 18 and 19). The differences in the amount of piroxicam detected in the stratum corneum at time zero may have been a result of the differences in both the release and skin penetration kinetics during the 3 hr occlusion. As can be seen from Table 26, the percent piroxicam released from the gel base after 6 hr was in the range of 14.43 to 28.61% which was only about two times lower than the percent absorption after skin stripping (range = 41.78-78.10%). This was in contrast to diclofenac which showed very much slower release rate, with the percent released at 6 hr about 6-10 times smaller than the percent absorbed at

the same period (Table 25). The data for piroxicam thus indicated that the release of the drug from the vehicle may not be sufficiently slow to be considered as the rate-limiting step for the overall percutaneous absorption. Therefore, difference in the release kinetics may contribute significantly to the overall piroxicam absorption. This may explain the lack of correlation observed between the amount of piroxicam detected in the stratum corneum at time zero and the *in vitro* release parameters. Furthermore, since the rate constants were different, the ranking of the four products with respect to the amount of piroxicam remaining in the stratum corneum at later times (1, 3 and 6 hr after removal of the excess gel) were also different from that at time zero (Table26). This would also explain the lack of the *in vitro-in vivo* correlation observed at these time points.

The new born pig skin, on the other hand, is a heterogeneous system of which the permeability has been claimed to approximate that of human beings better than rabbits, rats, and guinea pigs (Barry, 1983). However, data in Table 22 showed that the *in vitro* pig skin model failed to give correlation with the *in vivo* results. Although the new born pig skin is a heterogeneous system. Further, measurements of the thickness of the new born pig skin specimens gave values ranging between 230-300 micron (Appendix V). On the other hand, Barry et at. (1983) reported that the thickness of the stratum corneum of human forearm is in the range of 10-15 micron, the values which are 20-23 times smaller than the new born pig skin. Since the permeability coefficient is inversely proportional to the membrane thickness, the permeability of piroxicam through the thin stratum corneum of human

Table 25 : Comparison of percent released and average percent percutaneously absorbed of piroxicam in four gel products

Product	*In Vitro* release study	*In Vivo* percutaneously absorbed	
	Average percent released at 6 hr	Average percent absorbed at 3 hr	Average percent absorbed at 6 hr
A	14.43	43.59	52.36
B	28.61	71.72	78.10
C	22.13	29.88	41.78
D	16.04	57.28	68.03
mean ± SD	20.30 ± 5.59	50.62 ± 15.56	60.07 ± 13.98

Table 26 : Comparison of percent released and average percent percutaneously absorbed of diclofenac in four gel products (Samitamarn, 1995)

Product	*In Vitro* release study	*In Vivo* percutaneously absorbed	
	Average percent released at 6 hr	Average percent absorbed at 3 hr	Average percent absorbed at 6 hr
A	10.85	79.39	89.72
B	8.96	77.55	87.12
C	11.63	71.66	78.75
D	8.32	84.91	85.94
mean ± SD	9.94 ± 1.35	78.38 ±4.73	85.38 ±4.07

forearm could be much higher than through the new born pig skin (Feldman, 1967). So the result from *in vitro* permeation through new born pig skin was no correlation with *in vivo* skin stripping and *in vivo* pharmacological tests

Therefore, better *in vitro* models need to be developed which are capable of accurately describing the products' *in vivo* performance as well as distinguishing various formulations. Apart from the appropriate selection of membranes, the type of buffer used as a receptor fluid may also have a profound effect on its release/permeation profile. It is possible that the pH, ionic strength, type and concentration of buffer may significantly affect the solubility, dissolution rate and consequently the release/permeation characteristic of a drug from a topical preparation. The receptor phase is usually water, isotonic saline or pH 7.4 buffer. Other solvents can be used if the solubility of the drug is problem (Hadgraft, 1989). During the *in vitro* studies, isotonic phosphate buffer pH 7.4 was used as a receptor fluid in all experiments. Modification of the receptor fluid and other experimental conditions may lead to the *in vitro* release/permeation models which give better correlations with the *in vivo* results. However, such studies may require extensive experimental moreover, and presently, there is no best *in vivo* model that could be used as a reference for correlation. Thus, attention had to be shifted toward the evaluation of various *in vivo* models since they may be provide a more direct and better means to assess product efficacy and topical bioavailability.

4. Correlation Studies between *In Vivo* Skin Stripping and *In Vivo* Pharmacological test data

Since no correlations were observed between the *in vitro* release/permeation parameters and the *in vivo* parameters from both the skin stripping and the pharmacological tests, correlations between the *in vivo* skin stripping and the *in vivo* pharmacological studies were further tested at 5% significance level. As can be seen from data in Table 27, no significant correlations were observed between the amount of piroxicam detected in the stratum corneum at various times and the two pharmacological parameters under study, i.e. the ear weight difference and the size of blue spot ($p > 0.05$). Other *in vivo* skin stripping parameters such as the rate constants and percent absorbed at 1, 3 and 6 hr also did not correlate with the ear weight difference. However, it is interesting to note that there were significant correlations observed between the percent piroxicam percutaneously absorbed at 1 and 3 hr and the size of blue spot following intravenous injection of pontamine sky blue into the rats ($r = 0.9940$ and 0.9502; $p < 0.05$; Figure 24 and 25)

According to this pharmacological model, the deeper the penetration of the NSAIDs into the skin, the better is its activity to reduce the histamine-induced vascular permeability, as demonstrated by the reduction in the size of the blue spot. As previously stated, the rat skin was sensitized with intradermal injection of histamine, leading to increased vascular permeability and leakage of fluid into the extracellular spaces, This can be easily observed with the use of a color indicator like pontamine sky blue, a water-soluble dye which spreads rapidly into the leakage area. Therefore, the control rats on

Table 27 : Correlation test of *in vivo* skin stripping and *in vivo* pharmacological studies

***In Vivo* studies**	***In Vivo* studies**	**Correlation coefficient (r)**	**P-Value**
Amount in St. corneum weight at 0 hour	Ear weight difference	0.9285	$p > 0.05$
	Size of blue spot	0.6274	$p > 0.05$
Amount in St. corneum weight at 1 hour	Ear weight difference	0.7578	$p > 0.05$
	Size of blue spot	0.1269	$p > 0.05$
Amount in St. corneum weight at 3 hour	Ear weight difference	0.2975	$p > 0.05$
	Size of blue spot	0.4686	$p > 0.05$
Amount in St. corneum weight at 6 hour	Ear weight difference	0.5360	$p > 0.05$
	Size of blue spot	0.4602	$p > 0.05$
Rate Constants over 6 hour	Ear weight difference	0.4014	$p > 0.05$
	Size of blue spot	0.8447	$p > 0.05$

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Table 27 : Correlation test of *in vivo* skin stripping and *in vivo* pharmacological studies (continue)

***In Vivo* studies**	***In Vivo* studies**	**Correlation coefficient (r)**	**P-Value**
% Absorbed at 1 hour	Ear weight difference	0.4668	$p > 0.05$
	Size of blue spot	0.9940	*$p < 0.05$
% Absorbed at 3 hour	Ear weight difference	0.6844	$p > 0.05$
	Size of blue spot	0.9502	*$p < 0.05$
% Absorbed at 6 hour	Ear weight difference	0.6160	$p > 0.05$
	Size of blue spot	0.9446	$p > 0.05$
$AUC_{(0\text{-}6\ hr)}$	Ear weight difference	0.5837	$p > 0.05$
	Size of blue spot	0.5894	$p > 0.05$
Ear weight difference	Size of blue spot	0.3795	$p > 0.05$

* = significant at $\alpha = 0.05$

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Figure 24 Correlation between percent absorbed of piroxicam at 1 hr and the blue spot size of four gel products (r = 0.9940)

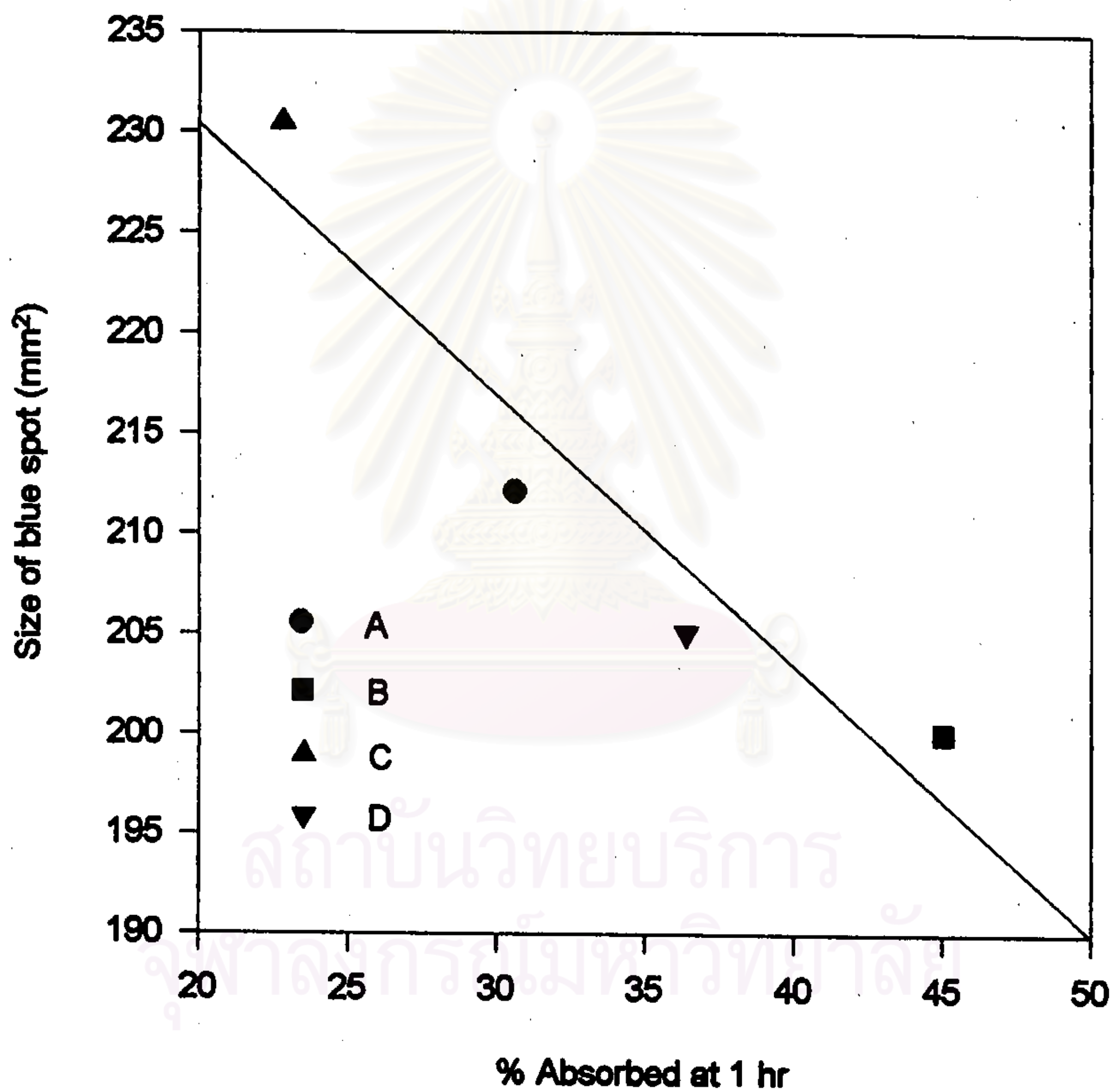

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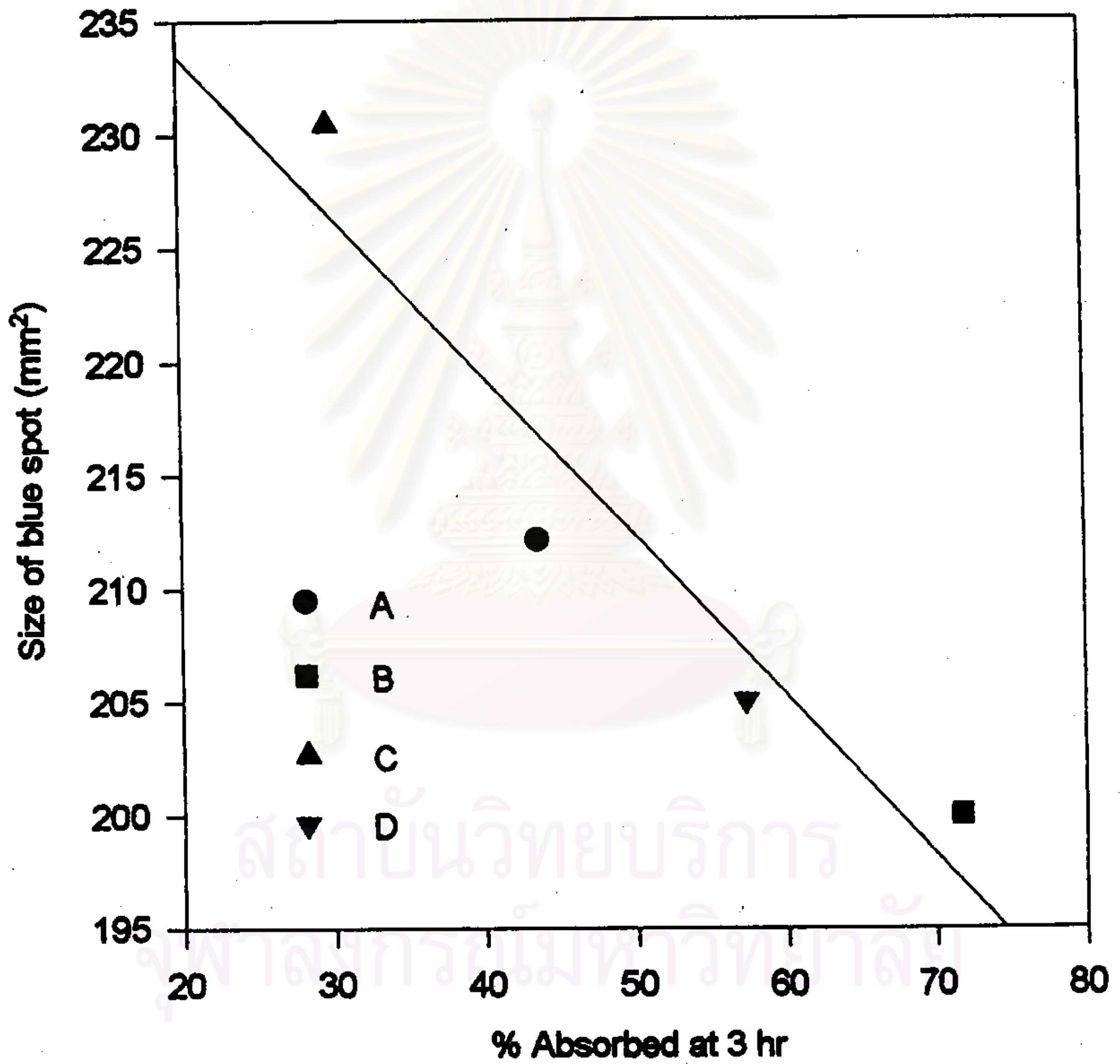
Figure 25 Correlation between percent absorbed of piroxicam at 3 hr and the blue spot size of four gel products (r = 0.9502)



which no drug was applied showed the largest blue spot following intravenous injection of the dye. The rank order of the four products with respect to the ability to reduce the size of blue spot is C < A < D < B. This ranking is roughly the same as that for the percent absorbed through the stratum corneum at 1 (C < A < D < B) and 3 hr(C< A < D < B), indicating a fairly good correlation between these skin stripping parameters and this pharmacological model. Product B which showed greatest percent absorbed, either at 1 or 3 hr, also showed the highest ability in reducing the size of blue spot. On the other hand, product C which showed the lowest percent of drug penetration was also the least effective in inhibiting vascular permeability. Products A and D had intermediate drug penetration and therefore, were moderately effective in reducing the blue spot.

Although the percent drug absorbed at 6 hr did not significantly correlate with the size of blue spot, the correlation coefficient was nearly close to the point of observing significance ($r = 0.9446$; $p = 0.0554$). The percent drug absorbed at 1 hour gave the best correlation results ($r = 0.9940$). This parameter is equivalent to the initial rate of percutaneous absorption or the absorption rate at the first hr. The percent drug absorbed at 3 hr gave smaller correlation coefficient ($r = 0.9502$) which was still significant although rather marginally. The observation of the smaller correlation coefficient value at later times might be explained by the profile of the drug loss from the stratum corneum (Figure 23). From this figure, it can be seen that all the four products demonstrated an initial sharp drop in the amount of piroxicam loss from the stratum corneum, indicating rapid percutaneous

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absorption during the first hr. The rate of drug penetration then gradually declined after 1 hr and appeared to reach saturation at about 6 hr. There was very small loss of piroxicam from the stratum corneum at 6 hr when compared to the amount remaining at 3 hr for all the four products, Saturation of drug loss from the stratum corneum observed at this time point has made it difficult to accurately determine the first order rate constant of percutaneous absorption from the overall profiles. At present, the reason for observing the saturation of drug penetration at 6 hr was not clearly known. However, it is possible that penetration through the stratum corneum of a lipophilic molecule like piroxicam could be slowed down by further processes of drug diffusion through the viable epidermis and dermis which are known to be more hydrophilic than the stratum corneum. Hadgraft (1989) suggested that the first step in the absorption process is partitioning of the drug in the outer layers of the stratum corneum. This region of the skin is largely lipophilic in nature, and the first step in absorption is facilitated for drugs which have a high lipid-water partition coefficient. After the drug has partitioned into the skin, it will be diffused through the stratum corneum as a result of the concentration gradient that exists. The route of penetration has been the subject of considerable debate, but current opinion suggests that the major pathway is through the intercellular channels. These are filled with a structured array of neutral lipids, and it is through these that the drug must diffuse. At the interface between the stratum corneum and the viable tissue, the drug must undergo a further partitioning step in order to reach its targets. The viable tissue is largely aqueous in nature, and transfer from the stratum corneum will be favored for NSAIDs which have hydrophilic properties.

Results of Samitamarn (1995) also showed similar profiles for diclofenac, in which loss of the drug from the stratum corneum appeared to slow down and reach saturation at about 6 hr, where more than 80% of diclofenac has penetrated the stratum corneum. Slower penetration of lipophilic drug through the viable epidermis and dermis may have resulted in accumulation of the drug in these layers, thereby causing the drug content in the stratum corneum to decrease more slowly. Nevertheless, more evidence is still needed to colloborate the above hypothesis.

Although the apparent first order rate constants (k) of the stratum corneum after stripping did not correlate with the size of blue spot, the correlation coefficients was relatively high ($r = 0.9149$; $p = 0.0851$) As previously mentioned, the values of k were obtained from linear regression of the plots of piroxicam remaining in the stratum corneum versus time over the 6 hr period. Saturation of the piroxicam penetration through the stratum corneum during 3 to 6 hr could lead to the calculated k values which did not correlate with the size of the blue spot. Another possible reason for lack of correlation could be that the drug penetration through the stratum corneum may not be simply described by the first order passive diffusion process. Skin composition is very complex, consisting of several layers and different cell types. The human skin comprises two distinct but mutually dependent tissues, the stratified, a vascular, cellular epidermis and an underlying dermis of connective tissue. At the bottom of the dermis lies the fatty, subcutaneous layer (Hadgraft, 1989). The skin, however, is a heterogeneous multilayer tissue consisting of stratum corneum, viable epidermis and dermis, and within

each layer it may recognize strata of different physiochemical properties. In percutaneous absorption, therefore, the concentration gradients develop over many strata in multilayer barrier. It is thus necessary to consider the diffusional properties in general of laminates, i.e. multilayer sandwiched together with each layer behaving as an isotropic field (Rongone, 1981). Furthermore, the formulations of each piroxicam gels used in this study were not known. Each product may contain certain "inactive ingredients" which may be able to temporarily alter the skin permeability. For example, alcohol and certain volatile oils often included in the topical preparations are known to cause changes in skin permeability. They have an irritant and rubefacient action, and volatile oil causes first a sensation of warmth and smarting (Reynolds, 1989a). In addition, the vehicle in formula may change the in tegrity of the skin and this will influence absorption (Rongone, 1981). This could be a major factor responsible for different percutaneous absorption kinetics of piroxicam gels observed in this study. Future studies, either *in vivo* or *in vitro*, are thus needed to elucidate the exact mechanisms of piroxicam percutaneous absorption from different formulations. Presently, only the percent drug penetrating the stratum corneum at 1 hr (the initial absorption rate) appeared to be the best skin stripping parameter which showed significant correlation with the drug's ability to reduce vascular permeability *in vivo*.

In contrast to the permeability model (size of blue spot), results in Table 27 indicated that there were no correlations whatever between the *in vivo* skin stripping parameters and the ear weight difference following

oxazolone-induced ear edema in mice. This could be partly explained by the differences in the nature of the experiments between the two pharmacological models. In the ear edema model, the irritant (oxazolone) was topically applied to the right ear of the individual mouse to induce edema. However, edema is just one of the early signs of the complex information processes. The effect of various piroxicam gels in reducing oxazolone-induced ear edema could be too superficial to accurately reflect the skin penetration ability of these products. It is the model of allergic contact-delayed hypersensitization reaction (Bouclier et al, 1989). In fact, Duncan's test results indicated that the four gel products were not different in suppressing ear edema (D < C < A < B). Tengamnuay et al (1995) have used the mouse ear edema model to evaluate different diclofenac diethylammonium gel formulations. They also found that all the tested diclofenac products were significantly effective over the control group. However, the model similarly failed to distinguish various diclofenac gel formulation despite their large differences in the release and percutaneous absorption characteristics as reported by Samitamarn (1995). It thus appeared that the mouse ear edema was less sensitive than the rat vascular permeability model in discriminating the piroxicam gel formulations.

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In the case of the latter model, passive cutaneous anaphylaxis can be elicited by an intravenous injection of antigen in animals previously sensitized intracutaneously with homologous or heterologous antibodies. It has been of great value in *in vivo* study on immediate hypersensitivity reaction (Bouclier et al, 1989). Histamine was intradermally injected to rats

to induce changes in vascular permeability. As a result, the extent of inflammatory reactions was more extensive and occurred in a deeper area of the skin as opposed to the ear edema model. The drug must be released from the gel base and penetrate to the deeper skin layers in order to be able to reduce the permeability of the blood vessels which are located in the dermis and subcutaneous tissue. Thus, the ability of piroxicam gels to reduce vascular permeability appeared to depend on their ability to penetrate the skin, leading to significant correlation observed between the ability of these products in reducing the size of the blue spot and the percent of drug initialy absorbed. The more rapidly the drug penetrated the stratum corneum, the greater was its ability to decrease vascular permeability.

5. Determination of Relative Bioavailability

Since there are several *in vitro* and *in vivo* methods in evaluating the efficacy of topical NSAID products, the main objective of this thesis was to find the most appropriate methods that can characterize or distinguish different piroxicam gel formulations. This was not an easy task since there was no best method that could be used as an absolute reference system for comparison. Systemic bioavailability determination based on measurements of plasma piroxicam has using high-performance liquid chromatographic (HPLC) techniques have been develop for piroxicam determination in plasma (Macek and Vacha, 1987; Milligan, 1992; Cerretani et al, 1993). The limit of quantitation of piroxicam for the assays ranges from 50 to 500 ng/ml (Boudinot and Ibrahim, 1988). However, the justification of using plasma drug concentration as a reference parameter to evaluate topical formulations

is debatable, particularly for NSAIDs which are reported to preferentially accumulate in muscles and joints following topical administration. Plasma drug levels may not truly reflect the drug concentrations in these tissues which are the desired target sites. Fourtillan and Girault (1992) suggested that the theoretical advantages of local application of piroxicam include targeting the site of action while minimizing systemic blood concentration and potential adverse effect. The lack of systemic adverse events may be related to the low plasma concentrations achieved after local application. Data from single dose pharmacokinetic studies confirm that systemic absorption of locally applied piroxicam is substantially lower than that of oral or intramuscular piroxicam. Thus, it appears that efficacy and tolerability data from clinical trials as well as data from single-dose pharmacokinetic studies are consistent with the proposed theoretical advantages of locally applied piroxicam gel. Following application of a single dose of gel, detectable concentrations of piroxicam were achieved in synovial fluid and synovial membranes 2 to 24 hr after the application. Throughout the sampling period, piroxicam concentrations in synovial fluid approximated those of plasma; however, at early time points, synovial fluid levels were sometimes higher and at later time points tended to be lower than those in plasma. Piroxicam concentrations in synovial membrane were higher than those in synovial fluid in the majority of cases.

Piroxicam plasma concentrations increases gradually during the course of multiple applications of piroxicam gel. The average steady-state plasma concentrations achieved were in range of 350 ng/ml, or about 5% of

those observed in subjects receiving oral or intramuscular piroxicam 20 mg daily. It is therefore suggested that the bioavailability of piroxicam via the percutaneous route relative to the amount bioavailability by mouth or by intramuscular injection is of the order of 0.14/4.76 or 3% (Fourtillan and Girault, 1992).

Selecting the most appropriate methods, therefore, had to rely upon correlations among different models. Five models have been tested in this study. They consisted of two *in vitro* diffusion models (drug release through cellulose acetate and drug permeation through new born pig skin); *two in vivo* pharmacological models (vascular permeability and mouse ear edema); and one method based on measurements of the drug content in the human stratum corneum following skin stripping *in vivo*. Only the results from the vascular permeability model significantly corrrelated with the skin stripping model. Therefore, these two models appeared to be the most appropriate methods in evaluating topical piroxicam formulations in terms of their *in vivo* percutaneous absorption characteristics and pharmacological activities. The relative topical bioavailabilities of the four gel products, based on comparison of the percent piroxicam absorbed at 1 and 3 hr and the ability to reduce the size of blue spot, are shown in Table 28 and 29, respectively. Product B was found to be the most effective with the greatest relative bioavailbility as compared to product A (reference product), whereas product was the least bioavailable. Although statistical result in Table 19 indicated that there was no significant difference in the percent absorbed at 1 hr, data at 3 hr implied that the four products could be statistically classified into three groups, i.e.

Table 28 *In vivo* topical bioavailability based on the percent absorbed through the stratum corneum at 1 and 3 hr of the three piroxicam gel products (B, C and D) relative to the reference product (A)

Product	**Relative Bioavailability %absorbed at 1 hr**	**Relative Bloavailability %absorbed at 3 hr**
A	100.00	100.00
B	146.90	164.53
C	74.33	68.55
D	118.66	131.41

$$\text{Relative bioavailability} = \frac{\text{\% absorbed at 1 hr of sample}}{\text{\% absorbed at 1 hr of reference}} \times 100\ \%$$

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Table 29 *In vivo* topical bioavailability based on the ability to reduce the size of blue spot of the three piroxicam gel products (B, C and D) relative to the reference product (A)

Product	Relative Bioavailability
A	100.0
B	135.48
C	46.10
D	120.74

Relative bioavailability =

$$\frac{\text{ability for reduce the size of blue spot of sample}}{\text{ability for reduce the size of blue spot of reference}} \times 100\ \%$$

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the most bioavailable group (B), the intermediate group (A and D), and the least bioavailable group (C). Duncan's test results on the size of blue spots also roughly indicated similar grouping, although the comparison was made on four products plus control.

As previously discussed, correlation was observed with the percent drug absorbed at 1 and 3 hr. These are parameters associated with the rate of drug penetration. On the other hand, no correlations were observed with the parameters related to the extent of percutaneous absorption. These are $AUC_{0\text{-}6hr}$ or the area under the drug content in the stratum corneum versus time curve and the amount of piroxicam remaining in the stratum corneum at various time points. Determination of the relative bioavailability by comparing the extent of absorption is therefore not relevant. Significant differences in the kinetics of drug penetration among the four products further disqualified the use of absorption extent. AUC can be compared to see the effect of different formulations on topical bioavailability only when the kinetics of drug loss from the stratum corneum are similar among these formulations. This is based on the same principle in determining systemic bioavailability, i.e. the area under the plasma drug concentration-time curve can be compared only when the elimination kinetics (loss of drug from plasma) are the same for all products when administered at the same dose.

The application of the *in vivo* skin stripping technique to determine topical bioavailability provides some advantages over the conventional method. The analysis of drug in the tape-stripped stratum corneum is very

simple and there are no interfering endogenous substances. The amount of drug in this layer is also substantial, thereby increasing the assay sensitivity. Furthermore, this technique is non-invasive in contrast to the procedures involved in the withdrawal of blood or synovial fluids. The subject compliance is therefore much greater than the typical systemic bioavailability study. Loss of the drug from the stratum corneum should confirm the occurrence of percutaneous absorption into deeper skin layers as well as the underlying tissues and possibly systemic circulation.

Due to the simplicity of the design, the technique is much more rapid and economical than the conventional bioavailability study. Skin stripping can be easily carried out. All the subjects participated in this study did not complain of any pain during skin stripping. In addition, the four products were well tolerated by the subjects. None of the volunteers developed any skin hypersensitivity or erythema and all of them completed the study. Consequently, it appears to be a very convenient technique for rapid screening of drugs for their *in vivo* percutaneous absorption and topical bioavailability.

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However, the major drawback of this technique is the lack of pharmacokinetic information which usually can be obtained from the plasma data. In addition, loss of the drug from the stratum corneum does not always guarantee systemic absorption. It merely supports that some kind of percutaneous absorption is going on. The exact target tissues or sites of action, whether they are muscles, joints, blood or any other tissues, must be

confirmed by direct analysis of the drug and its metabolites in that particular organ or fluid. Nevertheless, for the drug which is already know to be systemically absorbed or primarily accumulated in the tissues relatively close to the site of application such as piroxicam, it may be possible to employ the *in vivo* skin stripping technique to characterize its *in vivo* percutaneous absorption without the need to directly analyze the drug concentration in the plasma or target tissues

On the other hand, the animal models are mostly used for the evaluation of drug action since they often are the necessary tools for mechanistic studies leading to knowledge of the various events occurring during skin inflammation. The increasing need for the proof of ethical use of animals has already resulted in the establishment of a variety of regulations for the control and type of animal models used in pharmacology. If it is obvious that *in vitro* assays using cultured cells, isolated cells or isolated enzymes can resolve certain ethical problems, it is also obvious that *in vivo* method s are indispensable for establishing dose ranges and potential clinical utility. Nevertheless, we must be cautious in making casual extrapolations of the animal data to humans. Results from the animal studies have to be interpreted very carefully (Bouclier et al, 1989).

The above conclusions were based on the available data obtained in this study. More studies should be carried out in the future to apply these two methods on a larger scale. More piroxicam gel products should be tested to confirm the above findings. In addition, other methods of evaluation must be

developed in order to establish the method that best differentiates the product's *in vivo* performance and can be used as a reference system in evaluating topical bioavailability of piroxicam and other NSAIDs.

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