Chapter IV

Results and discussion

1.1 Determination of Percent Labeled Amount of Diclofenac Diethylammonium

The purpose of this part of study was to standardize the topical diclofenac diethylammonium gel products with respect to the percent labeled amount prior to further *in vitro* and *in vivo* permeation studies. The assay results are shown in Table 1 and their method of calculation is provided in Appendix III. From this table, it is apparent that all the four products contained an equal amount of diclofenac, with the average percent labeled amount for each product close to 100%. 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 diclofenac diethylammonium.

Table 1: Percent labeled amount of diclofenac diethylammonium gels

Product	Percent labeled amount
A	99.01 %
B	97.29 %
C	97.42 %
D	97.65 %

1.2 *In Vitro* Release Studies of Diclofenac through Synthetic Membranes

These experiments were conducted to evaluate the *in vitro* release characteristics of four diclofenac diethylammonium gel products using the diffusion apparatus and different synthetic membranes. The four products (A, B, C and D) 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 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. Two synthetic membranes, namely cellulose acetate and Durapore® membranes, were used in this study to evaluate diclofenac release from the gel products. Both are porous membranes (pore size = 0.45μm). 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 these membranes. However, the two membranes differ in terms of their polarity. Cellulose acetate membrane is relatively hydrophilic whereas Durapore® membrane is a hydrophobic membrane made from polytetrafluoroethylene (PTFE). The porous membranes, particularly cellulose acetate, are commonly used in the *in vitro* drug release studies to prevent dispersion of the formulation into the receptor fluid. As a result, they mainly act as a support and not as a diffusion barrier.

Theoretical Background of *In Vitro* release from Vehicle

There are several identifiable processes that, theoretically, basic for the study of the release kinetics of drugs from the vehicle for the case in which release from the vehicle is rate-limitting (Higuchi, 1961).Higuchi depicted the situation which the vehicle is initialy saturated with solute, with excess solute uniformly suspended as tiny particles. The important assumption for derivation of the time dependency of release is Q (the total concentration) of drug is much greater than Cs (The solubility of drug in the vehicle).

The equation describing the release of solute was

$$M_t = \frac{\sqrt{2DCs\,(Q - C_S)t}}{2}$$

where D is the diffusivity (cm^2/sec) of the drug in the vehicle.

And the rate of release is

$$\frac{dMt}{dt} = \frac{1}{2}\sqrt{\frac{D(2Q-Cs)Cs}{t}}$$

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

$$Mt = \sqrt{2QDCst}$$

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

$$\frac{dM}{dt} = \sqrt{\frac{QDCs}{2t}}$$

From this equation predicts that the rate of drug release is proportional to the reciprocol 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 (Higuch,1967). 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 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 diclofenac from the four commercial products through cellulose acetate and Durapore® membranes are presented in Figures 11 and 12, respectively. The numerical data are also given in Tables 2 and 3. 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 these figures, such plots give relatively linear relationships with the regression coefficients greater than 0.99 in most products (Appendices IV). On the contrary, Figures 14-17 show the zero order plots between cumulative amount of diclofenac released or permeated as a function of time. It can be seen from these figures that the lines were not linear in all products regardless of which porous membrane, cellulose acetate or Durapore®, was used. These results thus support the use of porous synthetic membranes for evaluating diclofenac 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 Figures 11 and 12 demonstrate linear relationship over the six-hour sampling period, it can be implied that the vehicle was controlling the drug release according to the Higuchi equation where the drug

Table 2: Diffusion data of four brands of diclofenac diethylammonium gels released through cellulose acetate membrane

Time (hours)	Amount of Diclofenac Released* (μg)			
	A	B	C	D
10 min.	349.87±57.58	200.67±31.97	401.75±17.36	106.10±31.38
0.5 hr.	526.19±57.84	408.95±17.38	636.25±38.85	198.04±22.54
1 hr.	695.66±57.09	568.70±10.54	830.24±42.57	310.78±12.56
1.50 hr.	814.57±50.60	721.37±16.84	988.84±60.45	470.21±27.12
2 hr.	922.15±47.43	836.99±10.63	1138.63±49.67	560.48±28.02
2.50 hr.	1033.85±60.04	956.82±7.57	1275.19±50.73	684.42±21.00
3 hr.	1166.17±70.68	1051.53±18.86	1413.97±53.67	766.86±62.32
4 hr.	1391.26±76.17	1251.62±50.76	1604.49±44.13	975.51±123.36
5 hr.	1628.65±82.50	1441.09±104.90	1805.52±36.80	1187.71±220.07
6 hr.	1888.70±51.99	1560.61±34.41	2023.98±46.27	1448.17±306.34

* n = 3, mean ± SD

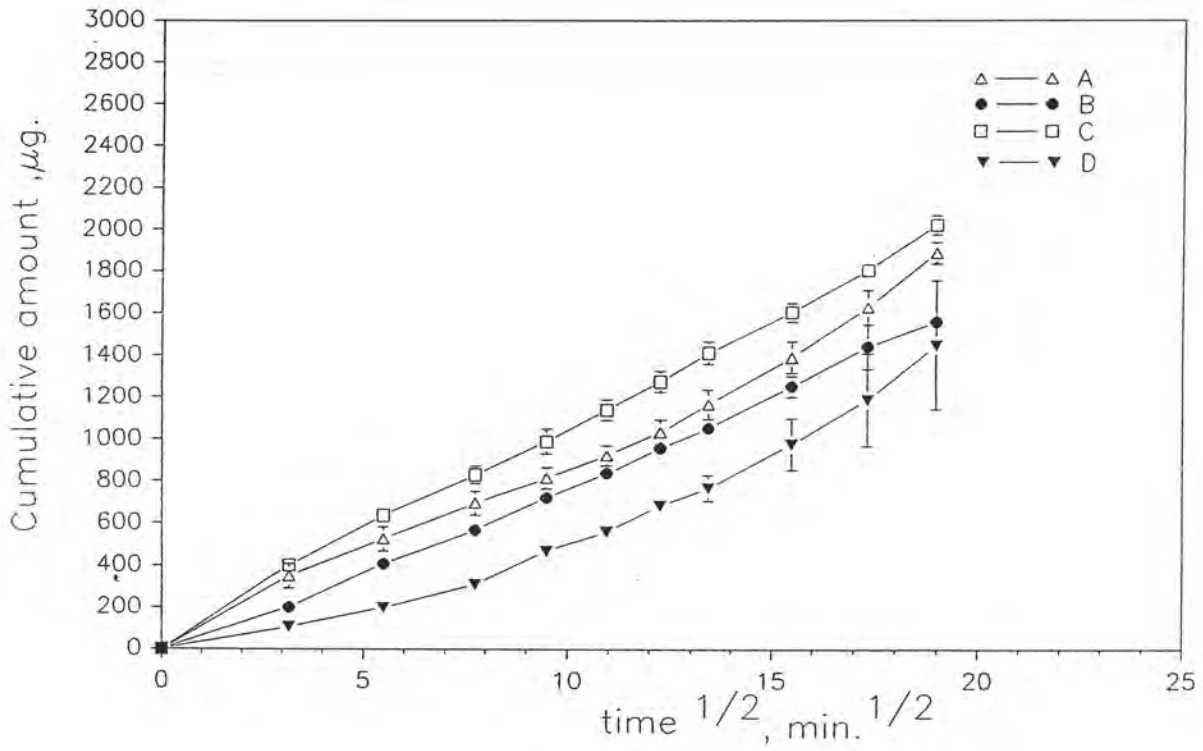

Figure 11: Release profile of four diclofenac diethylammonium gel products through cellulose acetate membrane
(Value = mean ± SD , n = 3)

Table 3: Diffusion data of four brands of diclofenac diethylammonium gels released through Durapore® membrane

Time (hours)	Amount of Diclofenac Release* (μg)			
	A	B	C	D
10 min.	155.32±35.63	45.15±1.20	152.41±9.97	57.97±3.00
0.5 hr.	274.11±39.31	100.76±4.02	250.57±13.95	162.06±8.17
1.0 hr.	407.90±39.08	176.5 ±68.01	374.15±20.47	268.55±11.86
1.5 hr.	518.09±43.06	246.13±11.41	496.09±28.38	370.78±17.11
2.0 hr.	620.03±37.88	301.12±14.84	587.91±27.29	458.54±15.12
2.5 hr.	717.06±35.85	345.01±16.39	654.62±37.21	524.12±30.20
3 hr.	806.56±36.98	392.72±18.07	732.75±36.41	585.47±35.95
4 hr.	929.39±47.03	481.23±20.96	862.60±49.26	716.73±43.09
5 hr.	1059.70±38.35	566.09±24.49	987.80±37.78	776.43±77.60
6 hr.	1171.26±33.61	654.38±43.04	1052.67±80.67	934.35±356.50

* n = 3, mean ± SD

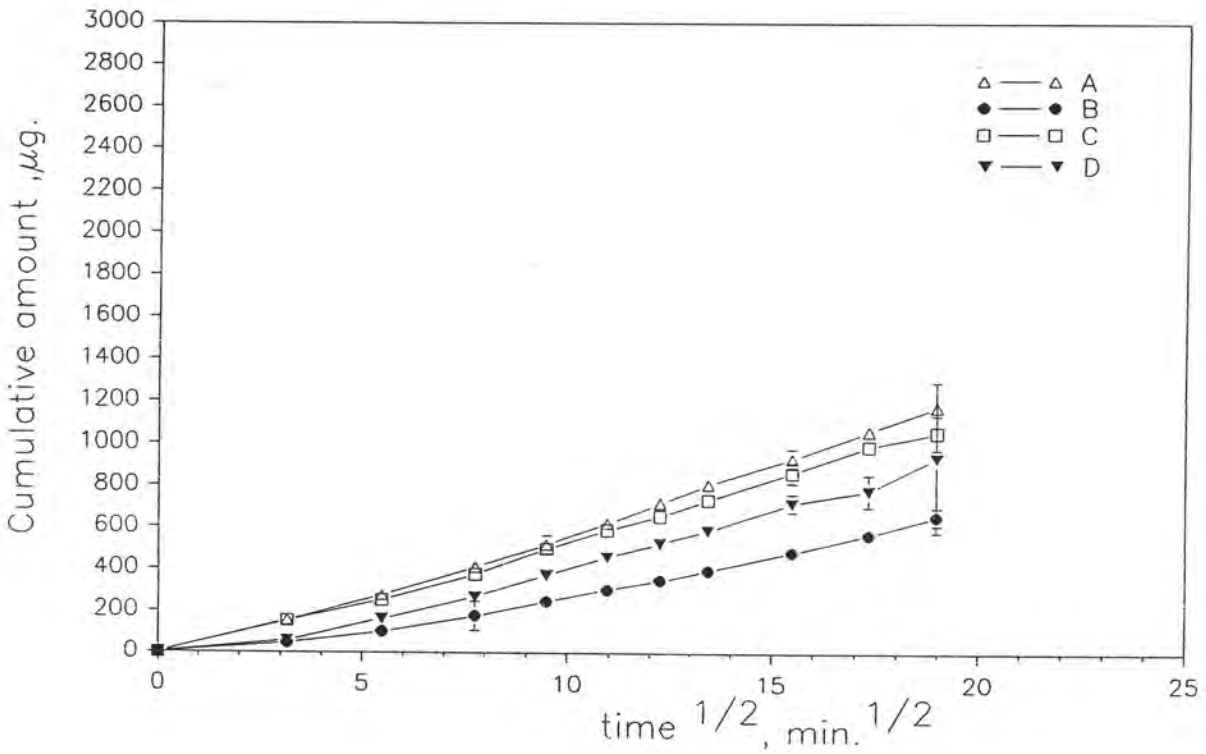

Figure 12: Release profile of four diclofenac diethylammonium gel products through Durapore® membrane
(Value = mean $\pm$ SD , n = 3)

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 Figures 14 to 17 even demonstrate the initial burst of drug released into the receptor fluid, with the slope being maximum in the initial period and gradually decreasing in both the cellulose acetate and Durapore® membranes. These observations again support the statement that the porous membranes did not play a significant barrier role in drug diffusion, although there were some differences in the release rate between cellulose acetate and Durapore® membranes (to be discussed in subsequent paragraphs). Therefore, the release characteristics of diclofenac from these products were governed almost entirely by the donor formulations. The release rate and cumulative amount in triplicate runs of the four products are also shown in Tables 4 for cellulose acetate membrane and Tables 5 for Durapore® membrane, respectively. The individual diffusion data and calculations of release rate are provided in Appendices IV.

The donor chamber of each diffusion cell contained 1.5 g of diclofenac gel product. This would give an excessive amount of drug to be penetrated and avoid depletion of the drug during study, thus maximizing the concentration gradient across the membrane. On the other hand, the receptor fluid consisted of 0.05 M pH 7.4 isotonic phosphate buffer. It has been reported that the approximate solubility of diclofenac diethylammonium in this buffer was higher than in Ringer-Locke physiological buffer (Chusanglertvichit, 1994). In addition, since diclofenac is a free acid with pKa about 4.07 (Maitani et al, 1993), the drug would be essentially fully ionized in this buffer. As a result, the isotonic phosphate buffer (pH 7.4) was selected as the receptor fluid so as to facilitate the dissolution and diffusion processes.

Table 4:Cumulative amount of diclofenac released from the four gel products through cellulose acetate and Durapore® membranes

Membrane	**Cellulose acetate membrane**		**Durapore® membrane**	
Product	Cumulative amount released* (μg)	%CV	Cumulative amount released* (μg)	%CV
A	1888.70±51.99	2.75	1171.26±33.21	2.84
B	1560.61±34.41	2.20	654.38±43.04	6.58
C	2023.98±46.27	2.29	1177.54±47.09	4.21
D	1448.17±306.3	21.15	934.35±56.50	6.05

*n = 3 , mean ± SD

Table 5: The release rate of diclofenac gel product through cellulose acetate membrane and Durapore® membrane

Membrane	**Cellulose acetate membrane**		**Durapore® membrane**	
Product	Release rate* ($\mu g/min^{1/2}$)	%CV	Release rate * ($\mu g/min^{1/2}$)	%CV
A	99.40±2.019	2.03	64.46±5.19	8.05
B	86.92±5.93	6.82	40.64±2.25	5.54
C	102.70±0.55	0.54	63.85±2.35	3.68
D	91.58±21.53	23.52	57.92±4.06	7.00

*n = 3 , mean ± SD

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

1.2.1 Statistical analyses of diclofenac 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 V) it is obvious that there was a significant difference in the cumulative amount of diclofenac released from the four products through cellulose acetate membrane at 6 hr ($p < 0.05$). A multiple range test (Duncan's test) was further applied to these data at the same significance level in an attempt to rank the four products with respect to their release characteristics. The results are shown in Table 6. As can be seen from this table, the ranking of the cumulative amount of drug released though this membrane, in an increasing order, was $\underline{D < B} < \underline{A < C}$. The two lines underneath the letters D and B and letters A and C signify that there was no significance difference between the two products in each pair ($p > 0.05$). For example, although product B released diclofenac to a greater extent than D, the difference was not significant ($p > 0.05$). Similarly, product C released the greatest amount of diclofenac but the value did not differ significantly from A ($p > 0.05$). Both A and C, however, significantly released diclofenac after 6 hr to a greater extent than B and D ($p < 0.05$). Based on the results observed with the extent of drug release, the four products can be roughly classified into two groups, i.e. a group with the greater amount of drug release (products A and C) and a group with the smaller amount of drug release (products B and D).

Table 6: Result from Duncan's test of *in vitro* release studies

Membrane	**Result** (at $\alpha = 0.05$)
Cellulose acetate membrane	
1. Cumulative amount released at 6 hour	$\underline{D < B} < \underline{A < C}$ (*p<0.05)
2. Release rate	$\underline{B < D < A < C}$ (p>0.05, NS)
Durapore® membrane	
1. Cumulative amount released at 6 hour	$\underline{B < D < A} < C$ (*p<0.05)
2. Release rate	$B < \underline{D < A < C}$ (*p<0.05)

NS = not significant at $\alpha = 0.05$

* = significant at $\alpha = 0.05$

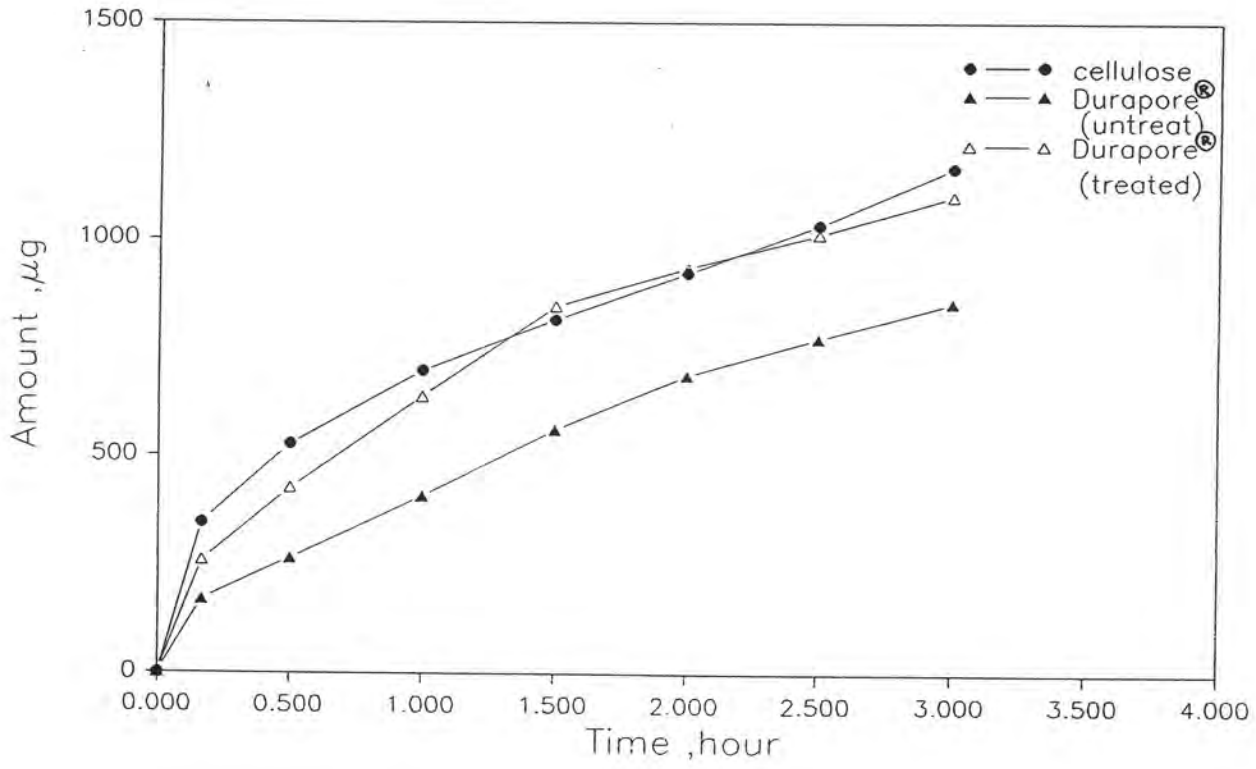

Figure 13: Comparison of permeation profile of standard diclofenac product through alcohol-treated Durapore® (Δ) ,untraeted Durapore® (▲) and cellulose acetate membrane (•)

On the contrary, when ANOVA was applied to the release rate data from the same membrane, there was no significant differences at 5 % level (Table 6, Appendix V). Nevertheless, the ranking order of the release rate was nearly similar to the cumulative amount, i.e. $\underline{B < D < A < C}$ (the line underneath the letters signify non-significance). Although there was a slight switch of the order between products B and D (release rate of B < D as opposed to the cumulative amount released of D < B), this was not significant at 5 %. Based on the values of their release rate, the four products can also be roughly classified into two groups similar to the data on cumulative amount released. For example, products B and D had similar release rates (86.92 versus 91.58 $\mu g/min^{1/2}$) but appeared to release diclofenac somewhat slower than products A and C (99.40 versus 102.70 $\mu g/min^{1/2}$). It can be seen that the release rates of A and C were also close to each other. As a result, a similar classification of the products may be assumed, i.e. a group with faster release rate (A and C) and a group with slower release rate (B and D).

One of the possible reasons for not being able to detect the significant difference with respect to the release rates, despite the highly significant difference in the extent of drug released, could be due to the nature of the square root of time plot which tends to flatten the initial slope. In addition, linear regression of these plots to obtain the slopes (and thus the release rates) may also be responsible for the observed non-significance. Since the calculation process involved the finding of a straight line which best fit the curve, the process of regressing the Y-values over the X-axis may give the slope values which were not significantly different among the four products.

1.2.2 Statistical analyses of diclofenac release through Durapore® membrane.

Similar statistical analyses, including ANOVA and Duncan's test, were also applied to the data obtained with Durapore® membrane. From Figures 11 and 12, it is obvious that diclofenac release through this membrane occurred to a smaller extent than through the cellulose acetate membrane in all the products studied. As previously stated, Durapore® membrane is highly hydrophobic. The membrane was therefore very difficult to wet in an aqueous environment and resulted in a much lower rate and extent of drug release (Tables 4-5). Preliminary experiments had shown that when Durapore® membrane was pretreated in methanol, diclofenac release from the gel products was greatly enhanced to the extent similar to that observed with cellulose acetate membrane (Figure 13). Therefore, these data further support the suitability of using porous membranes to study drug release from vehicle since both the cellulose acetate and Durapore® membranes, when appropriately wetted, did not act as a significant barrier against drug diffusion.

Statistical analyses (Table 6 and Appendices IV) revealed that there were also significant differences ($p < 0.05$) among the four products with respect to the rate and extent of diclofenac release through Durapore® membrane. When the Duncan's test was further applied, however, the ranking results were somewhat different from the cellulose acetate membrane. The ranking sequence of cumulative amount released was found to be B < D < A < C whereas the sequence of release rate was B < D < A < C. Similar classification of the four products into two groups could not be made as opposed to the case of cellulose acetate membrane. Since Durapore®

membrane is highly hydrophobic and was not pretreated with methanol in the actual experiments, incomplete wetting of the membrane during the release study was expected. It was likely that the membrane was not in full contact with the aqueous isotonic phosphate buffer in the receiver compartment. The extent of non-wetting was also not known. This could lead to the observed differences in the ranking results. Furthermore, the composition of the gel bases in each commercial product, except product D, was not available. Since these products contained unknown but varying amounts of alcohol and other additives, the extent to which the hydroalcoholic gel bases wetted the surface of hydrophobic Durapore® membrane could be highly variable. This could be an additional factor responsible for the variation in the ranking results when compared with the hydrophilic, easily wetted, cellulose acetate membrane.

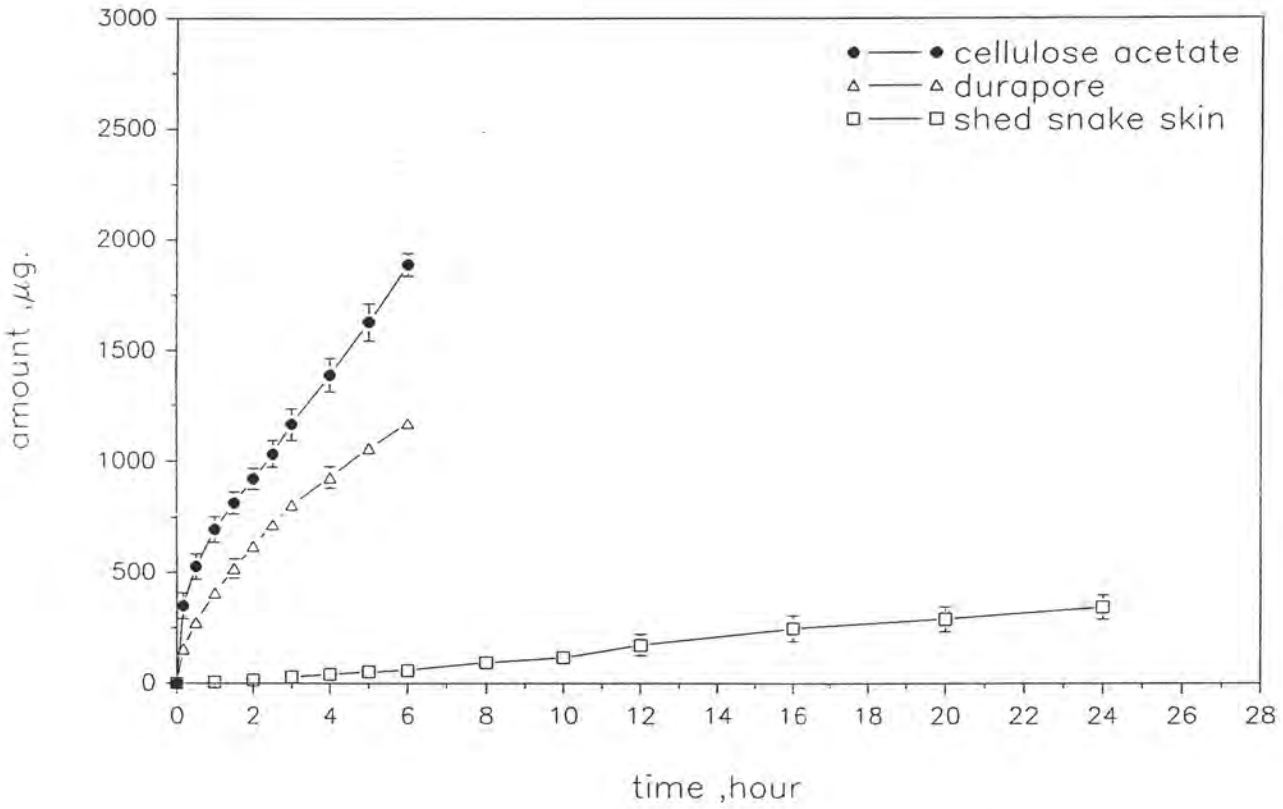

Figure 14: Diffusion profile of diclofenac diethylammonium (**Product A**) permeate through cellulose acetate membrane (•), Durapore® membrane (Δ) and shed snake skin (□) (Value = mean ± SD , n = 3)

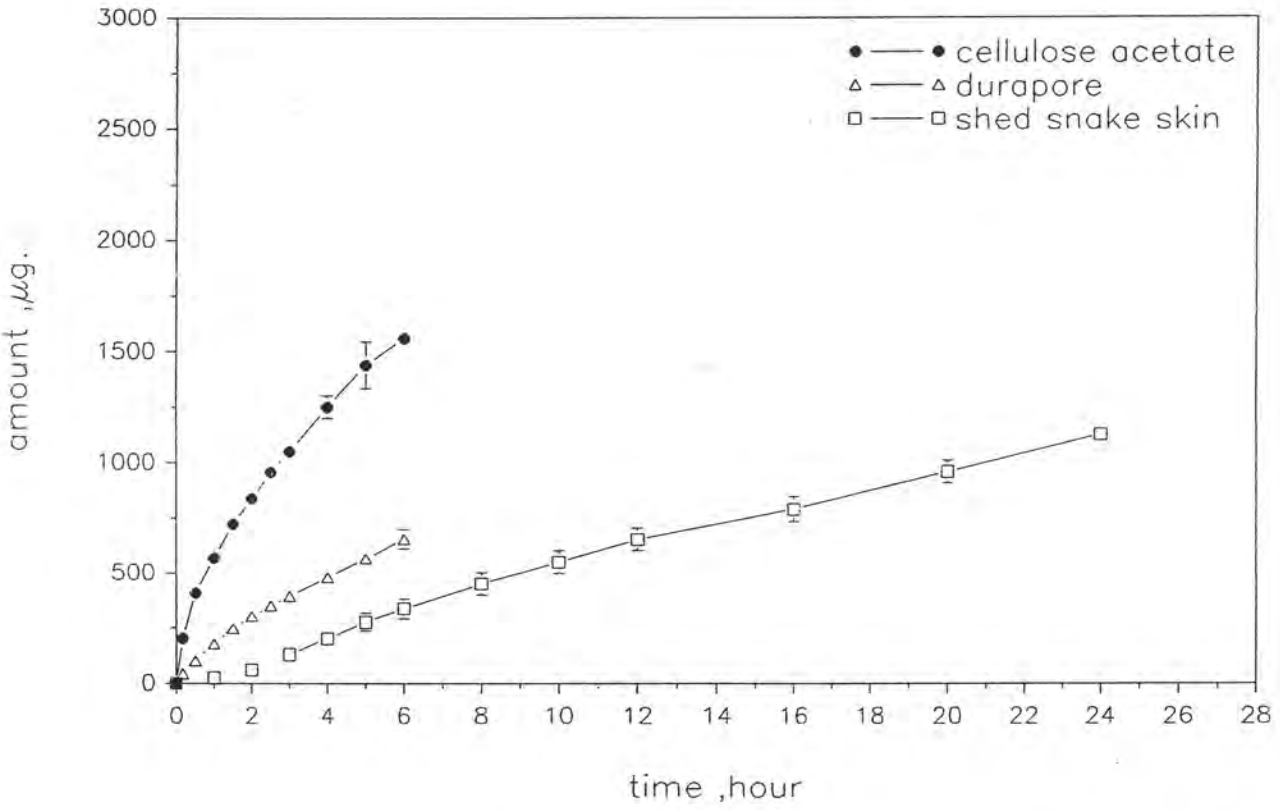

Figure 15: Diffusion profile of diclofenac diethylammonium(**Product B**) permeate through cellulose acetate membrane (•), Durapore® membrane (Δ) and shed snake skin (□) (Value = mean ± SD , n = 3)

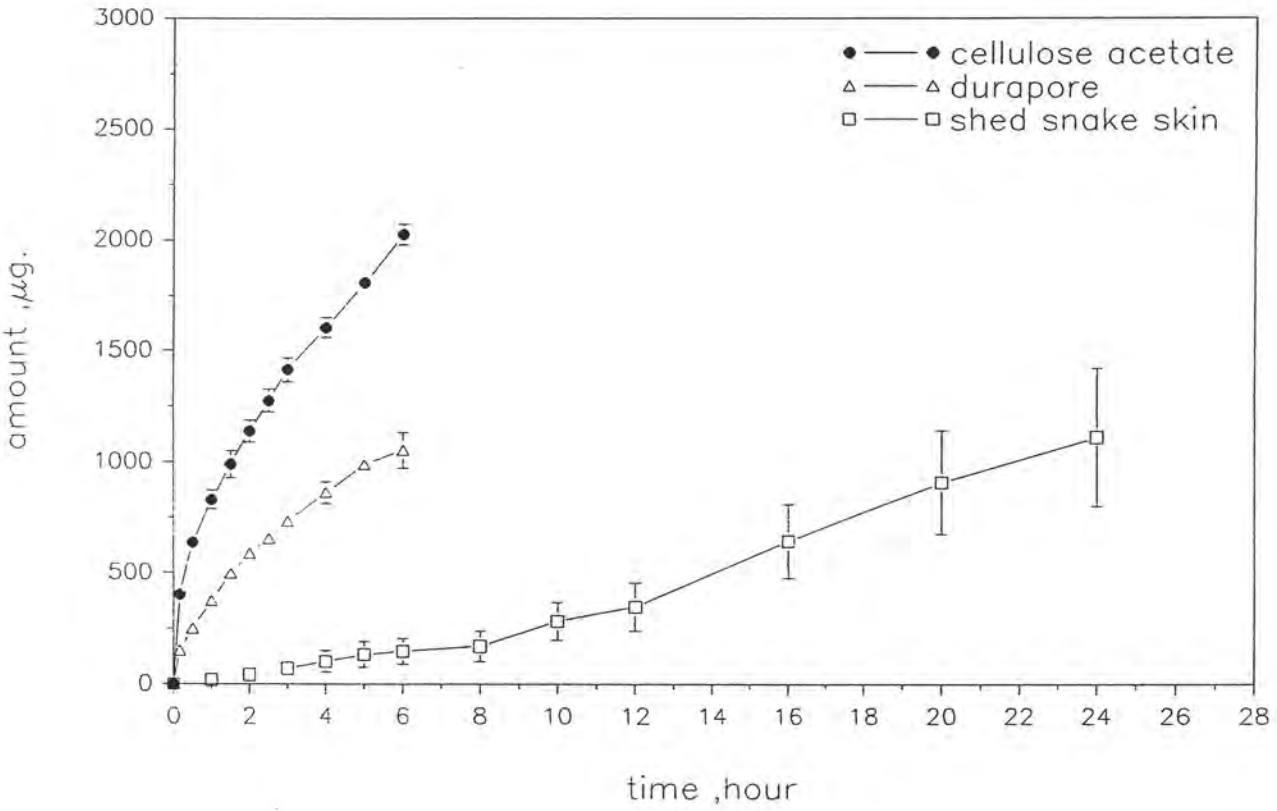

Figure 16: Diffusion profile of diclofenac diethylammonium(**Product C**) permeate through cellulose acetate membrane (•), Durapore® membrane (Δ) and shed snake skin (□) (Value = mean ± SD , n = 3)

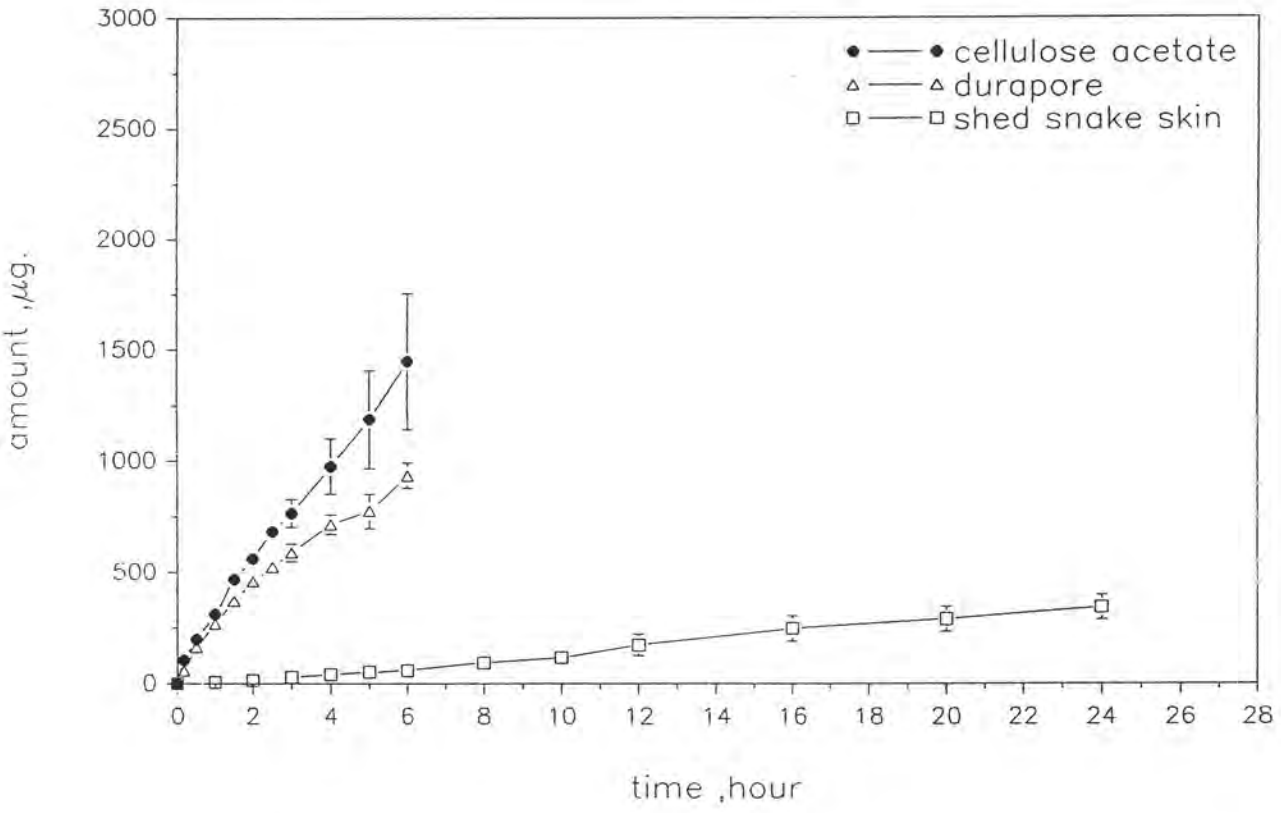

Figure 17: Diffusion profile of diclofenac diethylammonium (**Product D**) permeate through cellulose acetate membrane (•), Durapore® membrane (Δ) and shed snake skin (□) (Value = mean ± SD , n = 3)

1.3 *In Vitro* Permeation Studies through Shed Snake 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 the *in vitro* permeation of diclofenac diethylammonium 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. As previously stated, the shed snake skin was selected as a model membrane for the *in vitro* permeation studies because it consists of pure stratum corneum which is the most important barrier against drug diffusion through the skin, particularly in the transepidermal pathway.

This *in vitro* permeation study utilized shed snake skin specimens of *Elaphe obsoleta* which were obtained from Pata Zoo, Bangkok. The specimens were collected within 24 hours after the snakes shed their skin and kept in the freezer until the experiments. Before use, the specimens were thawed at room temperature and the dorsal area was then cut into a small round piece with the size slightly larger than the diffusion cell inner diameter. The membrane was subsequently immersed in pH 7.4 isotonic phosphate buffer for 12 hours before mounting onto the diffusion apparatus to allow for hydration and swelling (Itoh et al, 1991).

The average amounts of the four gel products penetrating through the shed snake skin at various times during the 24 hr-experiment are shown in Table 7. The cumulative amounts of drug penetrated at 24 hr as well as the steady-state fluxes are also summarized in Table 8 for each product. The

Table 7: Permeation data of four brands of diclofenac diethylammonium gels through shed snake skin.

Time (hours)	Amount of Diclofenac Permeated* (μg)			
	A	B	C	D
1	17.04±8.38	25.07±8.35	19.34±11.61	6.74±0.46
2	49.72±22.38	61.33±12.51	42.27±23.01	15.00±1.70
3	76.90±24.62	130.07±33.01	71.57±37.63	28.46±3.06
4	104.96±33.32	201.86±36.53	102.59±47.92	39.97±4.37
5	133.05±45.53	275.73±40.56	133.51±57.32	52.55±4.83
6	161.95±57.64	336.56±45.89	148.57±57.84	59.50±9.02
8	211.00±64.25	450.35±52.90	170.95±68.58	93.70±9.65
10	258.55±76.63	549.60±52.79	282.68±85.45	116.50±7.02
12	320.87±71.46	652.83±51.40	347.00±107.23	172.84±48.16
16	426.88±86.51	789.49±57.00	643.04±166.98	247.30±57.64
20	534.46±102.02	652.40±50.49	907.53±233.66	289.85±56.31
24	647.23±129.96	1125.85±37.69	1110.06±309.56	342.86±55.62

* n = 3, mean ± SD

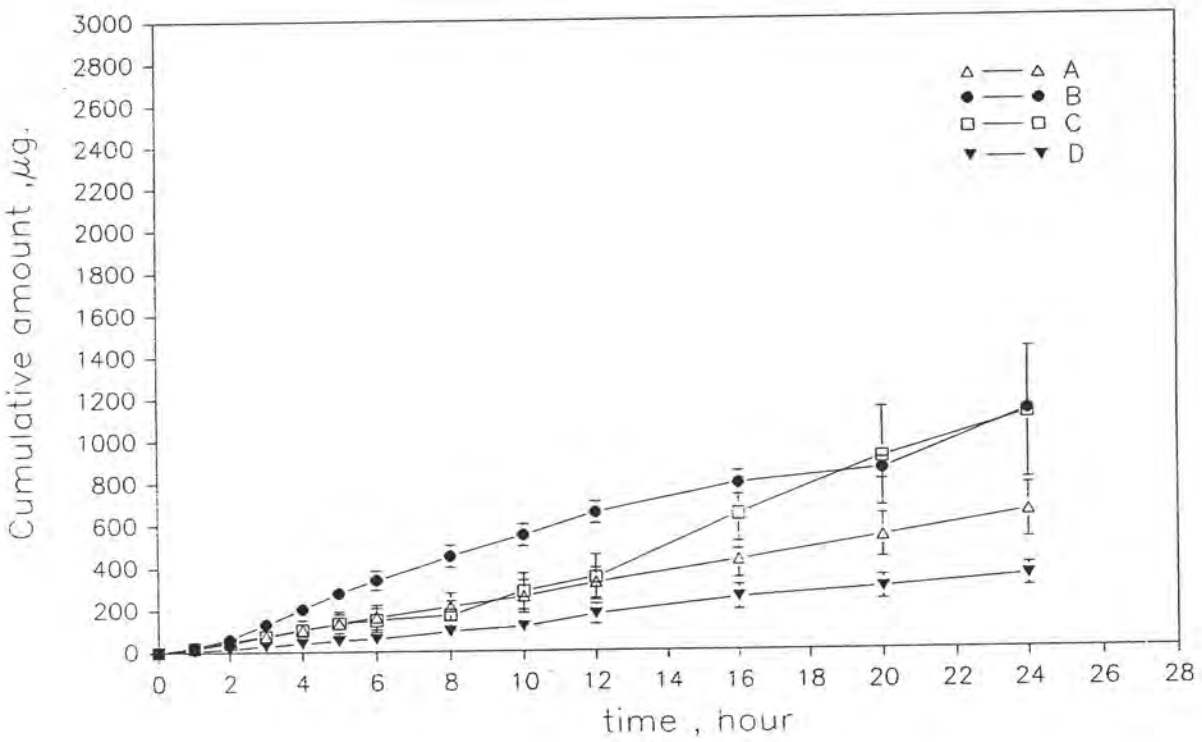

Figure 18: Comparison of permeation profile of four diclofenac diethyl-ammonium through shed snake skin from the four gel products (Value = mean $\pm$ SD , n = 3)

steady flux was obtained from the slope after linear regression of the terminal portion of the plot of the cumulative amount permeated versus time. Individual diffusion data, determination of the fluxes and permeation profiles for each product are provided in Appendix VI. From these data, the calculated regression analysis gave r^2 values which were greater than 0.99 in most of the experiments, indicating the establishment of steady state diffusion through this skin model according to the equation (Martin et al, 1983):

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

Fick's first law : The amount *M* of material flowing through a unit cross-section,*S*, of a barrier in unit time, *t*, is known as the flux, *J*

$$J = dM/S.dt$$

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 concentration in $\mu g/cm^3$ and x is the distance in cm of the movement perpendicular to the surface of the barrier

S is cross sectional area , cm^2

An important condition in diffusion is that of the steady state . Fick's first law equation give the flux in the steady state of flow. And in the seperated two compartments of a diffusion cell of cross-sectional area (S) and thickness,(h) and if the concentrations in the donor and the receptor sides are C_1 and C_2 ,respectively. The first law of Fick may be written as

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

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 low level (or as sink condition) ,therefore $C_1 >> C_2$ and $C_2 \rightarrow 0$ the steady state flux(J_{ss}) are described as below:

$$J_{SS} = PC_{SS}$$

where J_{SS} = Steady state flux of permeation, $\mu g/hr.cm^2$

P = Permeability coefficient, cm/hr

C_{SS} = Concentration gradient across the membrane barrier at steady state, $\mu g/cm^3$

Since the above equation was derived from Fick's first law, consistency of the data to this equation as demonstrated by the calculated regression coefficients should indicate the diffusion-controlled permeation of diclofenac through the shed snake skin membrane.

Permeation profiles or plots of cumulative amount of diclofenac permeated through the shed snake skin at various times are respectively shown in Figures 14 - 17 for the four products, in comparison with the cellulose acetate and Durapore® membranes. From these figures, it can be seen that the release and permeation of diclofenac through the shed snake skin was the slowest among the three membranes studied regardless of the products. The most rapid release of the drug was observed with hydrophilic cellulose acetate membrane, followed by the more hydrophobic Durapore® and the shed snake skin membranes. This is not unexpected since the shed snake skin is not a porous membrane in contrast to the cellulose acetate and Durapore® membranes. Rather, it consists of continuous layers of dead horny cells which are arranged in an orderly fashion and are rich in proteins and lipids. It thus acts as a formidable barrier against extraneous penetration of drugs and foreign substances. On the other hand, the two synthetic membranes did not play a significant barrier role against diclofenac diffusion. They 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 these membranes. Drug penetration through the shed snake skin, however, was a more complex process since the membrane played an 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 the shed snake skin. Since the shed snake skin is a non-living pure stratum corneum which is the most 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 1 hr before the steady state flux could be achieved (Appendix VI).

Table 8: Comparison of average cumulative amount and steady-state flux of diclofenac permeation from four gel products through shed snake skin

Product	Cumulative amount* (μg)	%CV	Steady state flux* (μg/min/cm^2)	%CV
A	647.23±129.96	20.08	17.67±5.25	29.71
B	1125.85±37.69	3.35	27.74±0.89	3.21
C	1110.06±309.56	27.89	41.46±11.27	27.18
D	342.86±55.62	16.22	10.12±1.72	17.00

*n = 3 , mean ± SD

Table 9: Duncan's test result on *in vitro* permeation of four diclofenac gel products permeate through shed snake skin

Shed snake skin (*E. obsoleta*)	F Value	P Value	Result
Cumulative amount released	14.8243	p = 0.0017	D < A < C < B (*P < 0.05)
Flux (Jss)	13.9232	p = 0.0021	D < A < B <C (* P < 0.05)

* = significant at α = 0.05

1.3.1 Statistical Analysis of Diclofenac Flux and Cumulative Amount Permeated through Shed Snake Skin

ANOVA was then applied to the data obtained from the permeation studies at 5 % level. The ANOVA results (Appendix VII) show that there were significant differences in the steady state flux and cumulative amount permeated at 24 hr among the four products. Graphical comparison of the permeation profiles is also given in Figure 18. The Duncan's test was further applied to these data and the results are shown in Table 9. It can be seen from this table that the ranking results, in an increasing order, for both the cumulative amount permeate is D < A < C < B and the flux is D < A < B < C. The lines underneath the letters D and A and letters C and B signify that there was no significant difference within each pair. Also, products D and A were significantly smaller than C and B at 5 % level as there was no line joining A and C. The statistical results from the shed snake skin, however, were different from those obtained from the porous synthetic membranes, particularly cellulose acetate membrane (D < B < A < C). The most likely explanation for the discrepancies in the ranking results observed between the cellulose acetate and the shed snake skin membranes was due to the barrier properties of the latter. The presence of an additional step (penetration of the drug through the rate-limiting membrane barrier) in the overall permeation process across the shed snake skin could lead to the differences in the permeation characteristics of diclofenac from each product. Nevertheless, the extent to which the barrier properties of the shed snake skin modified the overall permeation of diclofenac from these products and their ranking results is not clearly known. The data only implied that penetration of diclofenac through the shed snake skin occurred at a rate which was much slower than the release of the drug from the vehicle. Therefore, further *in vivo* experiments in humans should be conducted to test the correlation of the *in vivo* percutaneous absorption data with the *in vitro* release/permeation results. If any correlation has been found, either with

the *in vitro* release or permeation studies, the use of such *in vitro* tests should have some predictive values in assessing the topical bioavailability of these diclofenac gel products.

2. *In Vivo* Evaluation of Percutaneous Absorption Using Skin Stripping Technique

The purpose of this part of experiment was to develop a simple *in vivo* technique that could be used to quantitatively characterize the percutaneous absorption of an NSAID drug like diclofenac diethylammonium. 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 found in the blood was very low. To obtain valid results, the analytical techniques often employed complicate sample preparation steps and sophisticated instrumentation such as GC-MS which could be very expensive and time-consuming (Godbillon et al, 1984). It was thus very interesting to find an alternative in vivo technique which was able to distinguish, quantitatively, the topical bioavailability of various diclofenac gel products. Such the technique has been developed in this thesis based on the *in vivo* skin stripping approach.

2.1 Preliminary Study

There are many factors that need to be controlled during the *in vivo* skin stripping studies. Application time is one factor which may greatly influence the amount of drug percutaneously absorbed. Rougier et al (1992) have demonstrated that the total percutaneous absorption of drugs was directly proportional to the duration of their application. On the contrary, Akhter et al (1984) reported that maximizing the skin-drug contact time did not increase the amount of drug penetrating through the skin. Apparently, there should be an optimum application time for each drug which could result in the maximum drug release and penetration. This part of experiments was therefore designed to initially establish the optimum application time which could maximize the

initial amount of drug found in the stratum corneum. In this study the application time (interchangeably called occlusion time) was defined as the period during which the topically applied gel product remained in close contact with the skin surface under occlusion. After a pre-specified occlusion time (1, 3 or 6 hr), the excess gel was wiped off the skin surface by means of a cotton bud to terminate drug administration. The treated skin areas were then repeatedly stripped with a series of adhesive tape (Transpore®) to remove the stratum corneum for analysis of diclofenac content. Each treated area or "spot" was stripped ten times using ten separate tape strips. The ten sequential tape strips were then paired together and kept in five screw-capped culture tubes. For example, strips 1 and 2 were paired together and placed in tube no. 1, strips 3 and 4 in tube no. 2 and so on. Each pair was thus analyzed together for diclofenac content. The reason for this part of the experiments was to determine the appropriate sequence of tape strips that should correctly represent the actual amount of drug remaining in the stratum corneum. It was possible that some excess, unpenetrated drug may not be completely removed from the skin surface by wiping with cotton buds and thus remained on top of the stratum corneum. If the excess drug existed, it should be detected in the first few strips and be excluded from the analysis.

2.1.1 Determination of appropriate skin stripping sequence

After HPLC analysis, the data on the amount of diclofenac found in each pair of tape strips are shown in Table 10-12 for each of the three subjects participated. Plots of the amount found in each pair versus the number of tape strips are also shown in Figures 19-21. The analytical data are provided in Appendix VIII. It can be seen from these figures that the highest amount of diclofenac was always detected in the first pair of tape strips irrespective of the subject and the occlusion time. This finding indicated that the excess amount

Table 10: Preliminary results on skin stripping sequence in 3 subjects (Amount of diclofenac found in each pair of tape strips in subject number 1)

Occlusion time = 1 hour

Strip Number / Spot Number	Strip 1+2 Amount (μg)	Strip 3+4 Amount (μg)	Strip 5+6 Amount (μg)	Strip 7+8 Amount (μg)	Strip9+10 Amount (μg)
Spot 1	5.20	4.13	2.02	2.16	1.96
Spot 2	8.21	3.61	2.85	2.05	1.85
Spot 3	7.16	6.77	6.06	4.26	3.09
$\bar{X}$	6.86	4.84	3.64	2.82	2.30
SD	1.53	1.69	2.13	1.25	0.69

Occlusion time = 3 hours

Strip Number / Spot Number	Strip 1+2 Amount (μg)	Strip3+4 Amount (μg)	Strip 5+6 Amount (μg)	Strip 7+8 Amount (μg)	Strip9+10 Amount (μg)
Spot 1	18.22	7.15	5.62	4.63	3.03
Spot 2	15.53	8.94	8.98	5.52	4.06
Spot 3	23.65	10.36	12.89	5.05	3.28
$\bar{X}$	19.13	8.82	9.16	5.06	3.46
SD	4.14	1.61	3.64	0.45	0.54

Occlusion time = 6 hours

Strip Number / Spot Number	Strip 1+2 Amount (μg)	Strip3+4 Amount (μg)	Strip5+6 Amount (μg)	Strip7+8 Amount (μg)	Strip9+10 Amount (μg)
Spot 1	15.33	7.15	3.97	3.30	3.92
Spot 2	9.69	6.94	4.07	3.56	2.00
Spot 3	18.38	5.43	3.84	2.48	1.78
$\bar{X}$	14.47	6.51	3.64	3.11	2.57
SD	4.41	0.94	2.13	0.56	1.18

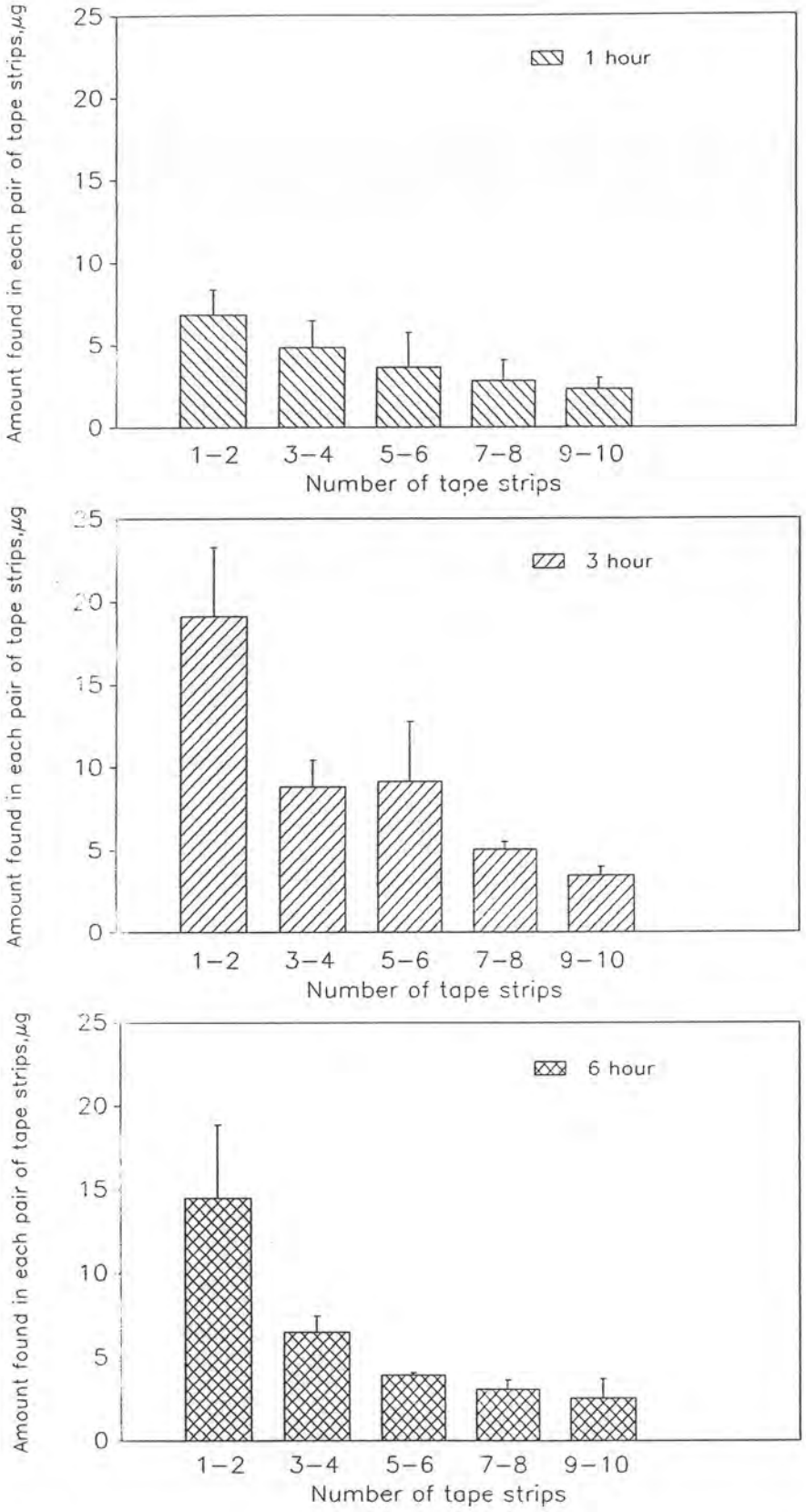

Figure 19: preliminary data on the average amount of diclofenac diethylammonium found in each pair of tape strips after 1 , 3 and 6 hour-occlusion : **Subject 1**
(Value = mean $\pm$ SD , n = 3)

Table 11: Preliminary results on skin stripping sequence in 3 subjects (Amount of diclofenac found in each pair of tape strips in subject number 2)

Occlusion time = 1 hour

Strip Number / Spot Number	Strip 1+2 Amount (µg)	Strip 3+4 Amount (µg)	Strip5+6 Amount (µg)	Strip 7+8 Amount (µg)	Strip9+10 Amount (µg)
Spot 1	6.49	4.42	3.02	2.43	2.61
Spot 2	5.86	3.76	3.05	2.83	2.75
Spot 3	8.09	3.87	2.73	3.00	3.66
$\bar{X}$	7.14	4.02	2.93	2.75	3.01
SD	1.15	0.35	0.18	0.29	0.57

Occlusion time = 3 hours

Strip Number / Spot Number	Strip 1+2 Amount (µg)	Strip 3+4 Amount (µg)	Strip5+6 Amount (µg)	Strip 7+8 Amount (µg)	Strip9+10 Amount (µg)
Spot 1	8.34	5.25	2.67	2.56	2.37
Spot 2	7.56	3.83	3.85	2.97	2.96
Spot 3	10.82	7.09	5.11	4.63	3.74
$\bar{X}$	8.913	5.39	3.88	3.39	3.02
SD	1.70	1.63	1.22	1.10	0.69

Occlusion time = 6 hours.

Strip Number / Spot Number	Strip 1+2 Amount (µg)	Strip3+4 Amount (µg)	Strip5+6 Amount (µg)	Strip7+8 Amount (µg)	Strip9+10 Amount (µg)
Spot 1	8.59	4.18	4.76	4.54	4.33
Spot 2	10.68	5.56	6.83	4.98	2.78
Spot 3	6.80	5.75	5.10	5.26	3.47
$\bar{X}$	8.69	5.19	5.56	4.93	3.53
SD	1.94	0.88	1.11	0.36	0.78

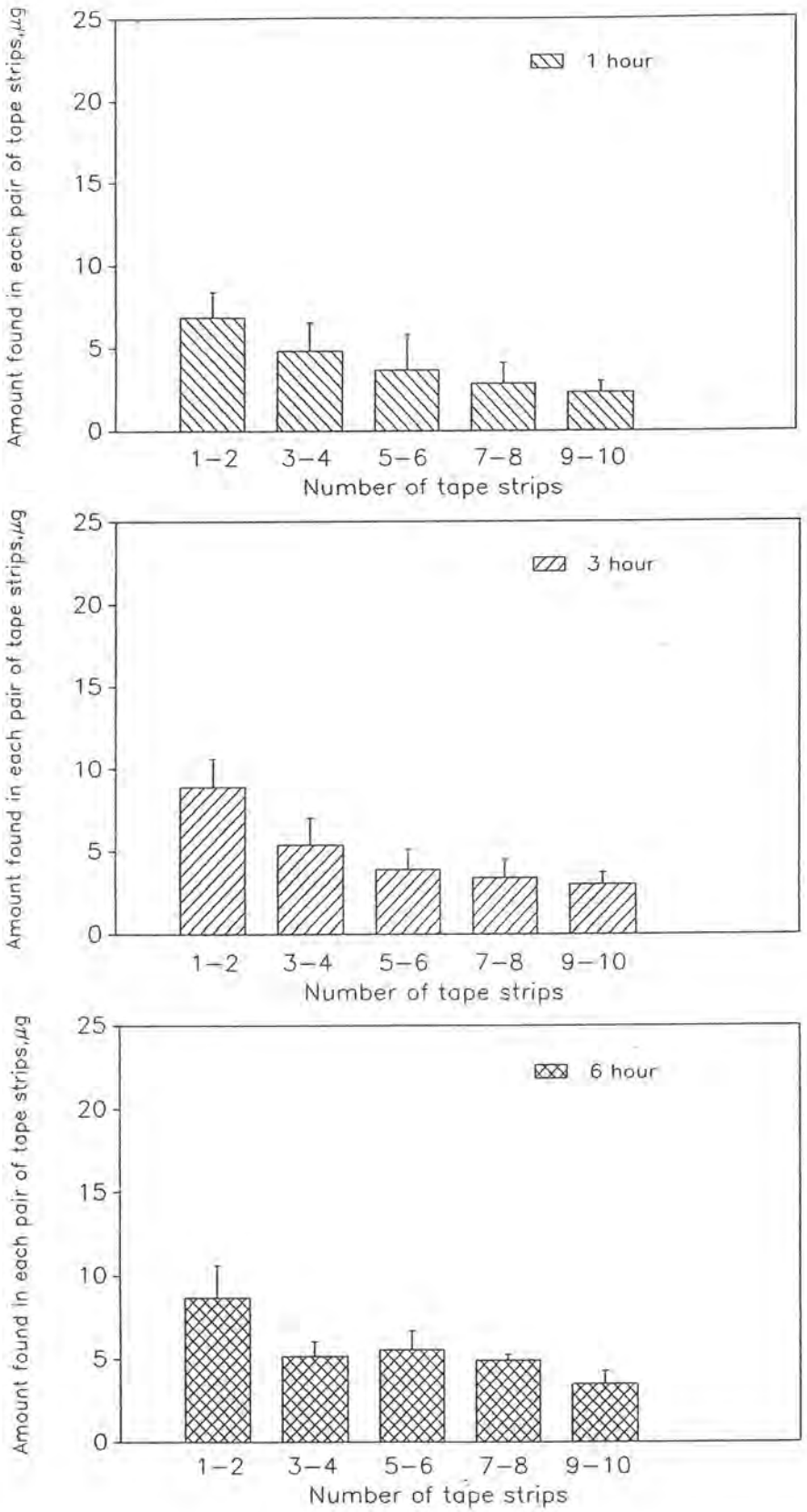

Figure 20: preliminary data on the average amount of diclofenac diethylammonium found in each pair of tape strips after 1 , 3 and 6 hour-occlusion : **Subject 2**
(Value = mean $\pm$ SD , n = 3)

Table 12: Preliminary results on skin stripping sequence in 3 subjects (Amount of diclofenac found in each pair of tape strips in subject number 3)

Occlusion time = 1 hour

Strip Number / Spot Number	Strip 1+2 Amount (µg)	Strip 3+4 Amount (µg)	Strip5+6 Amount (µg)	Strip7+8 Amount (µg)	Strip 9+10 Amount (µg)
Spot 1	5.04	1.96	1.27	0.84	0.95
Spot 2	3.87	2.10	1.30	0.84	0.69
Spot 3	6.63	2.03	3.13	2.22	1.38
$\bar{X}$	4.01	2.03	1.90	1.30	1.01
SD	0.40	0.07	1.06	0.80	0.35

Occlusion time = 3 hours

Strip Number / Spot Number	Strip 1+2 Amount (µg)	Strip 3+4 Amount (µg)	Strip 5+6 Amount (µg)	Strip 7+8 Amount (µg)	Stri 9+10 Amount (µg)
Spot 1	5.85	2.31	1.32	0.95	0.60
Spot 2	7.01	4.63	3.02	1.92	1.16
Spot 3	8.41	4.13	2.79	2.30	1.33
$\bar{X}$	7.09	3.69	2.38	1.73	1.03
SD	1.28	1.22	0.92	0.70	0.38

Occlusion time = 6 hours.

Strip Number / Spot Number	Strip 1+2 Amount (µg)	Strip3+4 Amount (µg)	Strip5+6 Amount (µg)	Strip7+8 Amount (µg)	Strip9+10 Amount (µg)
Spot 1	1.76	1.51	0.65	0.60	0.75
Spot 2	2.30	1.32	1.17	0.87	0.58
Spot 3	0.56	0.56	0.37	0.43	0.31
$\bar{X}$	1.54	1.13	0.73	0.63	0.55
SD	0.89	0.50	0.41	0.22	0.23

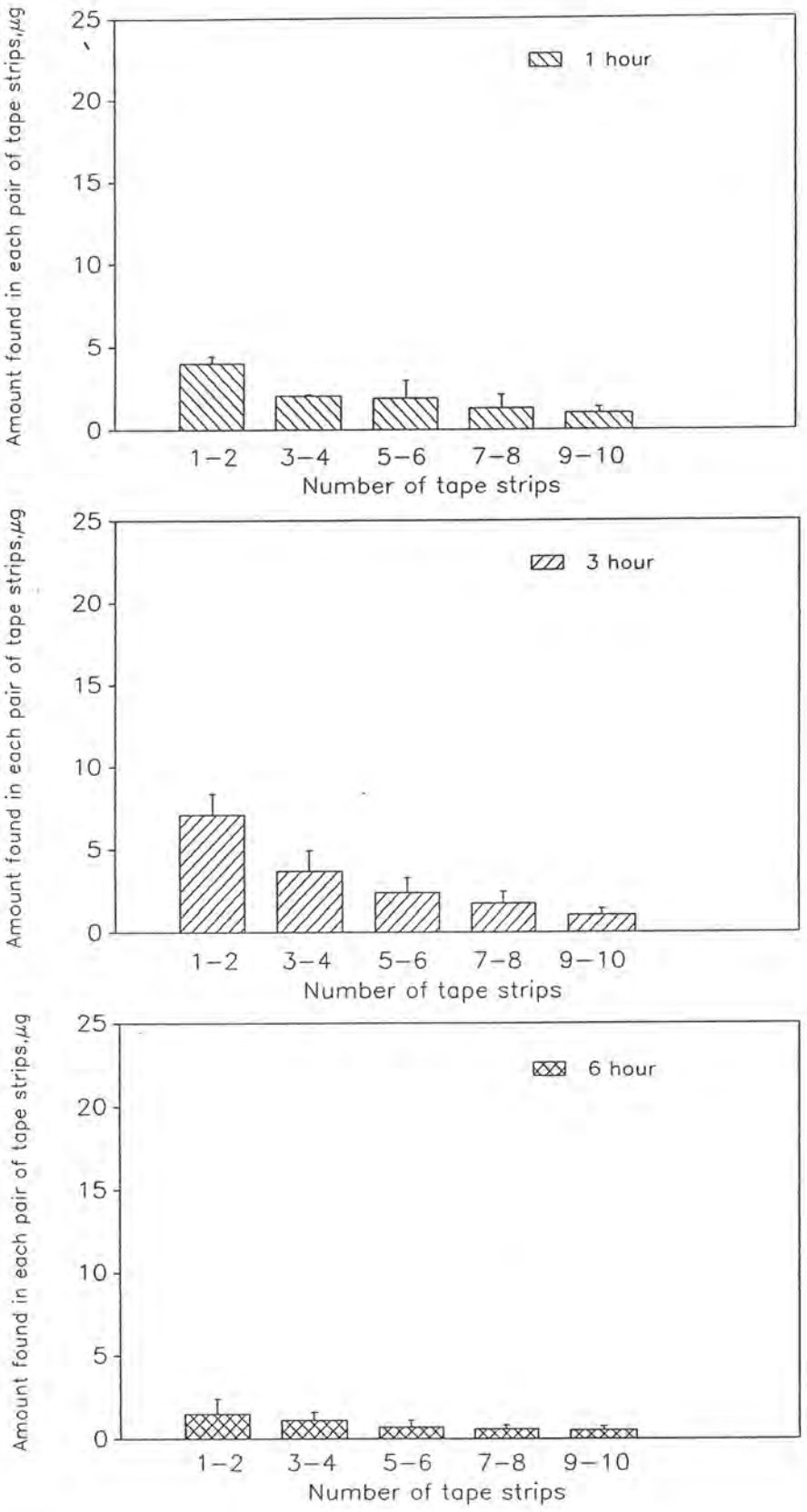

Figure 21: preliminary data on the average amount of diclofenac diethylammonium found in each pair of tape strips after 1 , 3 and 6 hour-occlusion : **Subject 3**
(Value = mean ± SD , n = 3)

subject and the occlusion time. This finding indicated that the excess amount of diclofenac gel product was not completely removed from the skin surface with the cotton buds but remained on top of the application spot. However, this unpenetrated residual amount was substantially removed off the skin surface during the first two strippings. The data were in agreement with the results of Pershing et al (1992) who reported that the first two strippings contained the excess amount of hydrocortisone which did not pass into the stratum corneum. Caron et al (1990) also found that the amount of hydrocortisone in the first two strips was significantly higher than the subsequent eight strips. However, the drug found in these uppermost layers did not represent the amount of diclofenac actually penetrating the lower stratum corneum. Rather, it was considered to be the unabsorbed drug remaining on the skin surface. As a result, the first two strips were discarded from the analysis. Only the remaining eight strips were combined for the total assay of diclofenac in the subsequent experiments.

In addition, it was noticed that the amount of diclofenac found in each pair of the later strips tended to decrease gradually, although not as sharply as the first and second pairs (Figures 19-21). The same authors (Caron et al, 1990) explained that this could due to the less tightly packed nature of the upper layers of the stratum corneum which may have taken the drug to a greater extent than the deeper layers, resulting in the analysis of later strips giving lesser amount of drug.

2.1.2 Determination of appropriate occlusion time

The same set of data were then reanalyzed by combining together the amounts of diclofenace diethylammonium found in strips no. 3 to 10 for each of the stripped spots in order to compare the effect of occlusion

time. The data are shown in Table 13 and graphically represented in Figure 22 for each of the three subjects. From this figure, it can be seen that the release and penetration of diclofenac through the stratum corneum appeared to be highest after three hours occlusion period. As a result, the application time of 3 hr was fixed in all the subsequent experiments since it was found to give the optimum amount of drug release and percutaneous absorption.

2.1.3 Effect of difference in application sites

One volunteer participated in this study in order to see if there were any differences in the amount of diclofenac found in the tape-stripped stratum corneum as a result of the difference in the application sites. As previously described in Chapter III, the subject's forearm was used as the application site in all the experiments. However, it was possible that stripping the stratum corneum from the different rows of spots of the same forearm could have an influence on the amount of the drug found in the tape strips. Likewise, the results obtained from the right and left forearms could also be different from each other. An experiment was thus designed to detect the effect of the difference in application sites by first dividing the subject's left forearm into five parallel rows. Each row was further divided into three application spots on which the drug administration and skin stripping took place (Figure 9 in chapter III). Product A (standard product) was applied onto each of the 15 spots in this 5 x 3 arrangement. Each spot was then occluded for 3 hr before skin stripping was started. Similar experiments were also conducted on the right forearm of the same subjects.

Following skin stripping, the amount of diclofenac in each spot was analyzed. The average value of the three spots within each row was also calculated as shown in Table 14 ANOVA was then applied to these data at 5 % level to test the the significance of the row effect. The results, along with the HPLC analytical data, are shown in Appendix IX. From the ANOVA table, it can be concluded that there was no significant difference among the five rows within the same forearm ($p >> 0.05$). This implied that, within the limited area of the forearm, application of the gel product to the different rows of spots did not have a significant influence on the amount of diclofenac found in the tape-stripped stratum corneum. Furthermore, when the data in Table 14 were statistically tested for the difference between the left and right forearms, no significant difference was also detected ($p >> 0.05$, Appendix IX). Thus, applying the drug to either the left- of right-handed side of the forearm should not have any significant effect on the amount of diclofenac found in the stratum corneum. The results from this part of experiments therefore provided an evidence that there was no interference in the percutaneous absorption of diclofenac due to minor differences in the application sites. Nevertheless, randomization of the application rows and the subject's forearm during drug application/skin stripping was always performed in all the experiments to guarantee that, if there was any influence due to the application sites, the effect would be randomly balanced.

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

Table 13: Cumulative amount of diclofenac (strip 3-10) found in the tape-stripped stratum corneum in each of the three subjects for effect of occlusion time

Subject 1

Occlusion time(hour) Spot No.	1	3	6
Spot 1	10.27	20.43	18.07
Spot 2	10.36	27.50	16.57
Spot 3	20.18	31.58	13.53
$\overline{X}$	13.60	26.50	16.0
SD	5.70	5.64	2.31
% CV	41.91	21.28	14.44

Subject 2

Occlusion time(hour) Spot No.	1	3	6
Spot 1	12.48	12.85	17.81
Spot 2	12.39	13.61	20.15
Spot 3	13.26	20.57	19.58
$\overline{X}$	12.71	14.20	19.18
SD	0.48	3.15	1.22
%CV	3.78	22.18	6.36

Subject 3

Occlusion time(hour) Spot No.	1	3	6
Spot 1	5.02	5.18	3.51
Spot 2	4.93	10.73	3.94
Spot 3	8.76	10.55	1.67
$\overline{X}$	6.24	8.82	3.04
SD	2.19	3.15	1.21
%CV	35.10	35.71	39.80

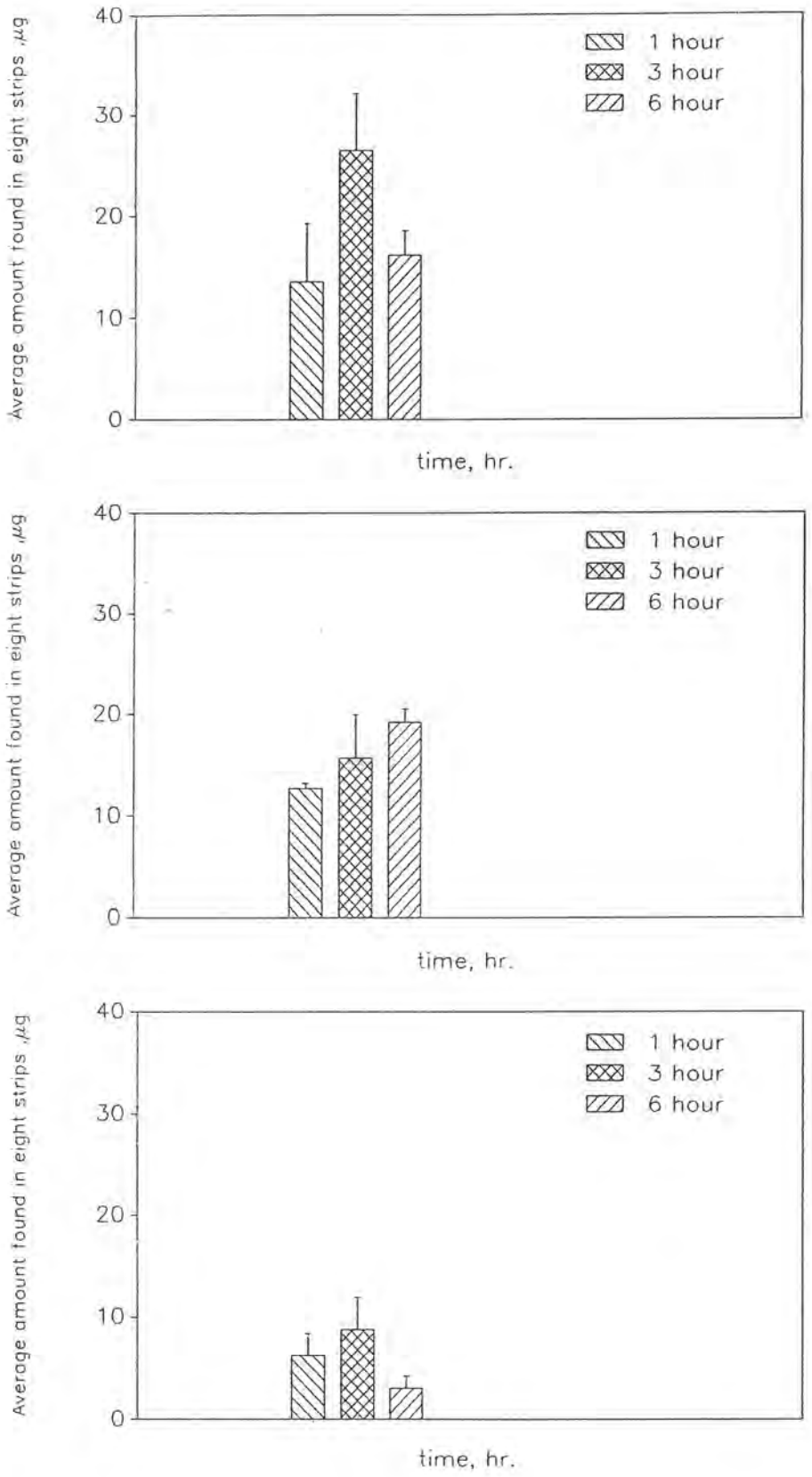

Figure 22: Effect of occlusion time on the amount of diclofenac found in the tape-stripped stratum corneum in each of three subjects (Value = mean $\pm$ SD , n = 3 strips)

Table 14: Effect of application site (row effect and left forearm versus right forearm) on the amount of diclofenac found in the tape-stripped stratum corneum.

Right Forearm

Spot No.	Row 1 Amount (μg)	Row 2 Amount (μg)	Row 3 Amount (μg)	Row 4 Amount (μg)	Row 5 Amount (μg)
Spot 1	13.27	13.00	12.05	18.35	20.44
Spot 2	11.56	13.43	12.24	16.62	16.95
Spot 3	12.95	20.35	13.11	16.17	18.12
X ± SD	12.59 ± 0.90	15.59±4.13	12.47±0.57	17.05±1.15	18.52±1.80
% CV	7.15	26.49	4.57	6.74	9.72

Left Forearm

Spot No.	Row 1 Amount (μg)	Row 2 Amount (μg)	Row 3 Amount (μg)	Row 4 Amount (μg)	Row 5 Amount (μg)
Spot 1	19.74	19.94	20.31	15.94	12.94
Spot 2	19.00	12.40	10.83	12.85	12.81
Spot 3	20.63	18.02	17.79	14.10	15.60
X ± SD	19.79±0.82	16.79±3.92	16.31±4.91	14.30±1.55	13.78±1.57
% CV	4.14	23.34	30.10	10.84	11.39

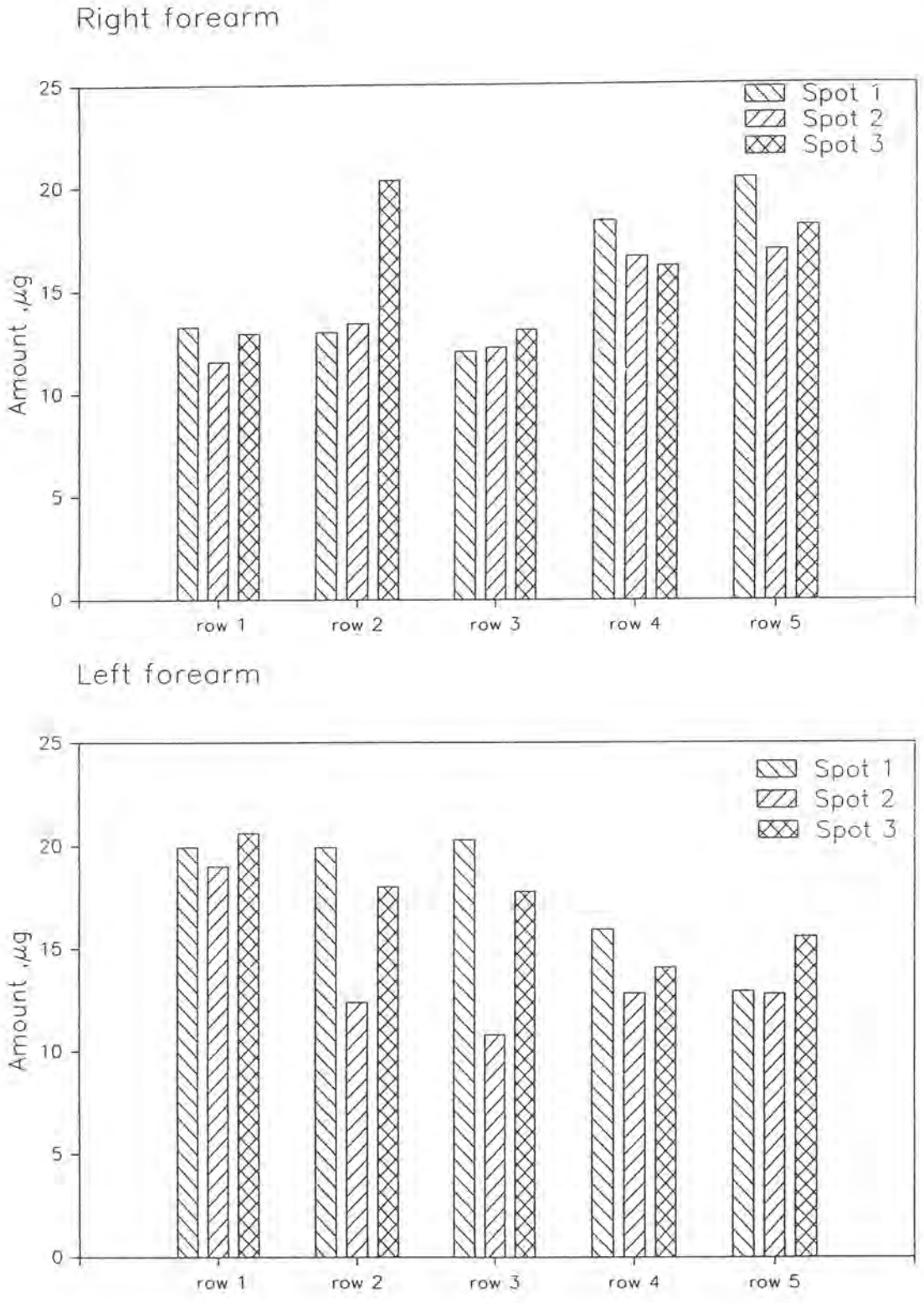

Figure 23: Effect of application site (row effect and left forearm versus right forearm) on the amount of diclofenac found in tape-stripped stratum corneum.

Following the preliminary study to ensure the suitability of the system, the *in vivo* skin stripping technique was then applied to the actual study in an attempt to evaluate the percutaneous absorption of diclofenac diethylammonium from commercial gel products and their topical bioavailability. Eight healthy volunteers (all females, age ranging from 24 to 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 one week washout periods. The treatment sequence was completely randomized for each subject.

Each product was topically applied to either the right or the left forearm of the individual subjects (whether the left or right forearm was used depended on random selection). Then the treated area was occluded for 3 hr to allow for the maximum release and penetration of the drug into the stratum corneum. The excess gel was then removed from each application spots with the use of cotton buds to terminate drug administration (and thus the drug release). As described in Chapter III, there were a total of eight application spots in the forearem of each subject (four rows of two spots each; Figure 10 of Chapter III). Immediately following removal of the excess gel, one row of the application spots was randomly selected for stripping to determine the initial amount of diclofenac in the stratum corneum, i.e. the amount at time zero. Another row was then randomly chosen for stripping at one hour after termination of drug treatment to determine the amount remaining at one hour. Consequently, the other two rows were randomly stripped at 3 and 6 hr, respectively, to determine the amount of diclofenac remaining in the stratum corneum at 3 and 6 hr. Loss of diclofenac from this layer upon termination of the drug application would imply the occurrence of percutaneous absorption to the deeper skin layers and underlying tissues.

The amounts of diclofenac found in the stratum corneum of each subject at time 0, 1, 3 and 6 hr following termination of drug application are shown in Tables 15-18 for the four products evaluated. The plots of average amount remaining in the stratum corneum as a function of time are provided in Figures 28 for the four products. The HPLC data and plots of the amount remaining versus time for the individual subjects are also given in Appendix X. From these figures it can be seen that there was a gradual decrease with time in the amount of diclofenac found in the tape-stripped stratum corneum in all subjects and products evaluated. This indicated that following the termination of drug administration, diclofenac was rapidly absorbed from the stratum corneum into the deeper skin layers. Since the stratum corneum is known to contain no enzymatic acitivities (Wertz et al,1988), loss of drug from this layer due to local metabolism was therefore unlikely. 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 diclofenac diethylammonium 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:

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

On the other hand, the processes of drug release and penetration were occurring simultaneously during the 3 hr application period. The two components both contributed to the overall percutaneous absorption. As a result, it was not relevant to determine percent drug absorbed during this period.

Table 15: Average amount of diclofenac remaining in the stratum corneum at various time : **Product A**

Average amount* (μg) Subject Number	**0**	**1**	**3**	**6**
1	20.49	8.51	3.61	0.41
2	15.65	7.72	3.92	1.84
3	24.18	8.36	3.88	2.52
4	19.75	10.20	5.71	4.31
5	7.61	3.09	1.96	1.31
6	19.80	3.00	1.78	0.23
7	7.16	3.06	2.34	0.76
8	19.88	4.75	3.27	1.47
mean**± SD	16.81±6.61	6.28±3.31	3.31±1.70	1.60±1.46

*average amount of two spot

* * mean of n = 16

Table 16: Average amount of diclofenac remaining in the stratum corneum at various time : **Product B**

Average amount*(μg) Subject Number	**0**	**1**	**3**	**6**
1	6.82	2.11	1.49	0.41
2	2.76	0.99	0.63	1.84
3	4.97	1.10	0.21	2.52
4	2.34	1.67	0.90	4.31
5	3.18	2.49	1.20	1.31
6	4.48	1.07	0.23	0.23
7	5.92	1.2	1.14	0.76
8	5.09	1.84	1.60	1.47
mean**$\pm$ SD	4.89 $\pm$1.74	1.62 $\pm$ 0.55	0.91 $\pm$ 0.61[A]	0.48 $\pm$ 0.42

*average amount of two spot

** mean of n = 16

[A] mean of n = 15

Table 17: Average amount of diclofenac remaining in the stratum corneum at various time : **Product C**

Average amount* (μg) **Subject Number**	**0**	**1**	**3**	**6**
1	9.20	13.41	10.48	8.87
2	12.57	6.36	6.36	2.67
3	21.20	3.05	5.08	1.75
4	23.72	16.14	0.79	1.06
5	14.11	9.32	2.17	4.55
6	15.21	7.20	6.49	6.27
7	18.84	6.16	0.95	1.22
8	6.07	1.65	1.33	0.78
mean**± SD	15.99 ± 5.50	7.53 ±5.42	4.20 ± 3.42	3.32 ± 3.10[A]

* average amount of two spot

** mean of n = 16

[A] mean of n = 15

Table 18: Average amount of diclofenac remaining in the stratum cournem at various time : **Product D**

Average amount*(μg) Subject Number	**0**	**1**	**3**	**6**
1	7.02	2.01	0.35	0.62
2	6.13	4.17	0.66	0.56
3	6.79	2.00	1.07	0.58
4	6.27	0.44	0.44	0.35
5	6.69	2.63	1.20	0.69
6	3.80	1.91	0.54	0.13
7	8.13	4.24	1.17	0.96
8	5.44	1.12	1.93	0.27
mean**± SD	6.28 ± 1.55	2.41 ± 1.73	0.92 ± 0.62[A]	3.32 ± 3.10

*average amount of two spot

** mean of n = 16

[A] mean of n = 15

Table 19: Average amount of diclofenac in four gel products remaining in the stratum corneum at various times
(each value = mean ± SD ,*n =16 ,**n = 15)

Time (hour) Product	0	1	3	6
A	16.81±6.1*	6.28±3.13*	3.31±1.70*	1.60±1.46*
B	4.89±1.74*	1.62±0.55*	0.91±0.61**	0.52±0.42*
C	15.99±5.5*	7.53±5.42*	4.20±3.42*	3.31±3.04**
D	6.28±1.55*	2.41±1.73*	0.92±0.62*	0.52±0.38*

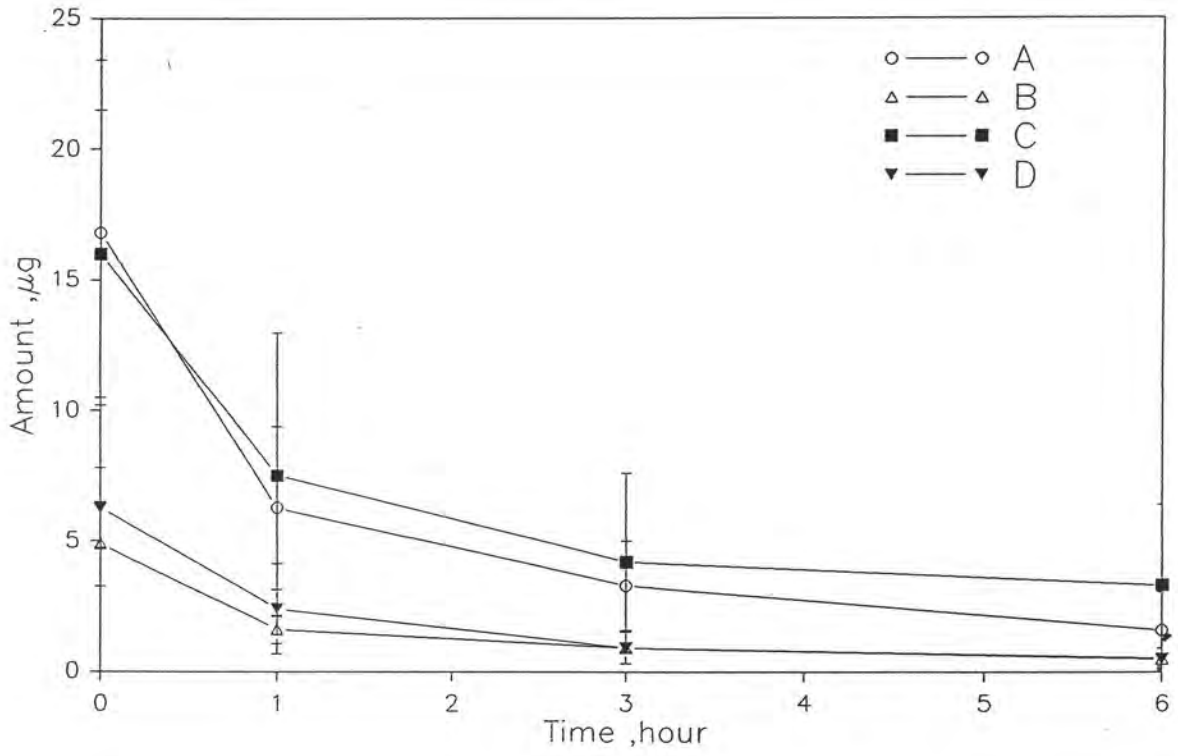

Figure 24: Average amount of diclofenac from four products remaining in stratum corneum at various times (Value = mean $\pm$ SD, n = 8)

The initial amount of diclofenac found at time zero was the highest with product A, the average value being 16.81 $\pm$ 6.61 μg (Table 15). Product C gave the second highest value of 15.99 $\pm$ 5.50 μg (Table 17), followed by product D at 6.28 $\pm$ 1.55 μg (Table 18). Product B gave the lowest initial amount at 4.89 $\pm$ 1.74 μg (Table 16). Since these values were the amounts found in the stratum corneum immediately after removal of the excess gel, it was possible that the differences observed among the four products may have been due to the differences in the drug release and/or penetration rates during the 3 hr occlusion period.

Two-way ANOVA was applied to test for any significant differences in the amounts of diclofenac found at time zero among the four products at 5% level. The statistical results are given in Appendix XI a. The data indicated that there was a strongly significant difference among the four products with respect to the initial amount ($F = 17.0848$; $p = 0.0001$). Duncan's multiple range test was further applied to rank the observed difference at 5% level. The results are also shown in Appendix XI a and summarized in Table 20. From this table, the ranking of the initial amount of diclofenac found in the tape-stripped stratum corneum, in an increasing order, was $\underline{B < D} < \underline{C < A}$. The same notation was applied in the data interpretation, i.e. the lines underneath the letters B and D and the letters C and A indicated that there was no significant difference between the two products within each pair ($p > 0.05$). Products A and C were significantly greater than B and D ($p < 0.05$) since there was no line joining the two pairs.

Table 20: Comparision of the amount of diclofenac remaining in the stratum corneum at various times.

Time (hour)	F Value	P Value	Result
0	17.0848	0.0001*	B < D < C < A
1	8.5980	0.0001*	B < D < A < C
3	5.2668	0.0042*	B < D < A < C
6	3.4577	0.0067*	B < D < A < C

* Significant at 0.05

Based on the Duncan's test result, the four products could be roughly divided into two groups, i.e one with the greater amount of diclofenac (A and C) and the other with the lesser amount of drug found in the stratum corneum (B and D). It is interesting to note that the ranking result was quite similar to that from the cellulose acetate membrane. From the data in Table 6, the ranking of the cumulative amount released though the cellulose acetate membrane at 6 hr was found to be $\underline{D < B} < \underline{A < C}$. Data from the cellulose acetate membrane indicated that the four products could be similarly divided into two groups, i.e. one with the greater amount of drug released (A and C) and the other with the lesser amount being released (B and D). It thus appeared at this stage that there could be some correlation between the *in vitro* release of diclofenac from the cellulose acetate membrane and the amount of drug found in the stratum corneum.

Two-way ANOVA was also applied to the data at 1, 3 and 6 hr. The results are given in Appendices XI b, c and d, respectively. As can be seen from these data, there were also significant differences among the four products with respect to the amount of diclofenac found in the stratum corneum at times 1, 3 and 6 hr ($p << 0.05$ in all cases). The Duncan's test was similarly applied to these set of data with the results shown in Table 20. From this table, the rankings at 1 and 3 hr ($\underline{B < D} < \underline{A < C}$) were found to be nearly identical to the result at 0 hr ($\underline{B < D} < \underline{C < A}$). Products A and C could be classified into the same group having the higher amount of diclofenac detected in the stratum corneum whereas products B and D were classified into another group with lower amount of drug found. On the other hand, the ranking results at 6 hr ($\underline{B < D < A} < C$) were somewhat different from those at time 0, 1 and 3 hr. The reason for such observed differences was not clearly known. It could be due to greater variability in the amount of diclofenac remaining at this later

hour which might have led to different statistical results. However, the data still showed the same ranking trend as those at the other time points.

Two-way ANOVA was utilized in analyzing the *in vivo* skin stripping data instead of one-way ANOVA. Since the *in vivo* experiment was of crossover design, the use of two-way ANOVA for multiple comparison of the four gel products in this study was analogous to the application of the paired Student's t-test in which only two products were compared (Sanford, 1991). Such the analysis would allow the subject effect (intersubject variability) to be separated from the total error term, thereby increasing the sensitivity of the test.

The percent of diclofenac 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 21 whereas the individual data are provided in Appendix XII. The results revealed that the percent drug absorbed during these periods was rather similar among the four products. At one hour after termination of treatment, the percent loss of drug from the stratum corneum (which implied percutaneous absorption) was in the range of 56.26 - 63.06% for the four products. After three hours more diclofenac had permeated through this layer and the percent penetration of the four products increased to 71.66 - 84.91%. At six hours, most of the products, exept product C, showed absorption extent greater than 85.1 %.

Two-way ANOVA was then applied to this set of data at 5% level. The statistical results are given in Appendix XII and also summarized in Table 21. From this table, it can be seen that there were no significant differences among the four products with respect to the percent drug absorbed at 1 and 3 hr ($p >>$

Table 21: Average percent of diclofenac percutaneously absorbed from four diclofenac gel products and their Duncan 's test results

Percent absorbed Time (hour)	A	B	C	D	Result
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	78.75±20.83	85.94±15.89	$p = 0.045$

* NS =not significance

0.05). At 6 hr the percent absorbed was found to be significantly different. However, the significance was only marginal as can be seen from the p-value which was very close to 0.05 (p= 0.045). This implied that diclofenac was percutaneously absorbed from the four products at about the same rate, at least during the first three hours. Such an implication also helped explain the observed similarities in the ranking results of the four products with respect to the amount of drug remaining in the stratum corneum at 0, 1 and 3 hr (Table 20).

Although the absorption rate appeared to be similar, the initial amount in the stratum corneum at time zero differed significantly among the four preparations ($p < 0.05$), with the ranking result after Duncan's test similar to that of the *in vitro* release through the cellulose acetate membrane. It thus appeared that the difference in the amount of diclofenac found in the stratum corneum could somehow be related to the difference in the release characteristics of the drug from the gel base.

2.3 Correlation Studies between *In Vitro* and *In Vivo* Data

Following an initial observation of the data from the *in vitro* and *in vivo* studies, there appeared to be some similarities in the ranking results of diclofenac release through cellulose acetate membrane (Table 6) and the amount found in the stratum corneum at time 0, 1 and 3 hr (Table 20). These ranking results indicated that the four products could be roughly divided into two groups with significant differences in the *in vitro* release and *in vivo* permeation characteristics. Products A and C gave better drug release from the cellulose acetate membrane than products B and D. Accordingly, they also yielded the higher amount of diclofenac in the stratum corneum at all time points. Products B and D, on the other hand, exhibited poorer drug release and

always gave a lower amount of drug found in the skin than the products A and C (Table 20).

A correlation test was further applied to these data to see if there were any significant correlations between the *in vitro* and *in vivo* parameters at 5% level. The results are summarized in Table 22, together with the correlation coefficients and the p-values. Two *in vitro* parameters, the cumulative amount released (or permeated) and the release rate (or steady state permeation flux), from each of the three membranes were tested for correlation with the amount of diclofenac found in the stratum corneum at 0, 1, 3, and 6 hr, respectively. As a result, there were a total of twenty-four correlation tests carried out. The details of each correlation are provided in Appendices XIII a, b, and c for cellulose acetate membrane, Durapore® membrane and shed snake skin, respectively.

The results from Table 22 indicated that only the *in vitro* release data from the cellulose acetate membrane significantly correlated with the *in vivo* skin stripping data ($p < 0.05$). None of the Durapore® and shed snake skin membranes gave any significant correlation with the amount of diclofenac remaining in the stratum corneum ($p > 0.05$). As previous stated, cellulose acetate is a hydrophilic porous membrane which does not have significant barrier properties against drug diffusion and simply acts as a support for the drug release from the topical vehicle. The observed significant correlation of the cellulose acetate data with the *in vivo* skin stripping results would therefore indicate that the release rate could be the rate-limiting step in the overall percutaneous absorption of diclofenac diethylammonium from these products.

As can be seen from Table 22, significant correlations were found between the release rate from the cellulose acetate membrane and the amount of drug found in the stratum corneum at 0, 1 and 3 hr ($r = 0.9548 - 0.9878$, $p < 0.05$). In addition, significant correlations were also observed between the cumulative amount of drug released through this membrane and the amount in the stratum corneum at 1 and 3 hr ($r = 0.9599 - 0.9851$, $p < 0.05$). The correlations are depicted graphically in Figures 25 to 29. Other correlation are from the area under the concentration-time curve (at 0-6 hour) of diclofenac diethylammonium products and the release rate of drog through cellulose acetate membrane ($r = 0.9785$) and area under the concentration-time curve of diclofenac with the cumulative amount released through cellulose acetate membrane ($r = 0.9731$).

It is interesting to note that the significant correlations were observed between the release rate through the cellulose acetate membrane and the amount found in the skin during the first three hours despite the non-significance in the ranking order of the release rate among the four products ($\underline{B < D < A < C}$; $p > 0.05$). On the other hand, while there was a significant difference among the four products in the *in vitro* cumulative amount released through this membrane ($\underline{D < B} < \underline{A < C}$; $p < 0.05$), the test for correlation was not significant between the *in vitro* amount released and the amount found in the stratum corneum at 0 hr ($r = 0.9326$, $p > 0.05$). However, the correlation became significant at later time points, i.e. at 1 and 3 hr.

The reason for no correlation between the *in vitro* amount released and the amount found in the skin at time zero was not clearly known in spite of the finding that the two parameters similarly showed significant rankings after Duncan's test. Variability of the data could be one factor responsible for the observed discrepancies. Similar groupings of the *in vitro* and *in vivo*

parameters after the Duncan's test into two pairs, i.e. a pair with better release and absorption (A and C) and a pair with poorer release and absorption properties (B and D), only provide a rough indication of possible *in vitro-in vivo* correlations. However, similar ranking results do not always guarantee the correlation. Likewise, the absence of significant ranking in these parameters by no means disproves the correlation possibility. A clear example is the *in vitro* release rate which still showed good correlation with the *in vivo* data despite the lack of significant differences among the four products (B < D < A < C). As a result, the correlation must be finally confirmed by a more relevant test such as the test for zero correlation as described in Appendix XIII.

When the *in vitro* parameters obtained from the Durapore® and the shed snake skin were scrutinized, none of them correlated with the amount of drug found in the skin at any time points. As can be seen from the data in Tables 6 and 9, the ranking results were much different from the cellulose acetate membrane, particularly with the shed snake skin. This was not unexpected, however. As previously mentioned, Durapore® is a lipophilic porous membrane which is difficult to wet in an aqueous medium like the phosphate buffer used as the receptor fluid. Preliminary studies have found that, after prewetting the membrane with alcohol, both the cumulative amount released and the release rate substantially increased to the level similar to the cellulose acetate membrane.

For the shed snake skin, the result in Table 22 showed the poorest correlations ($p > 0.05$ in all cases, $r = 0.1153 - 0.7646$). This could be due to the fact that permeation of diclofenac across this membrane is a result of two components, i.e. the release as well as the penetration steps. Lack of correlation between the *in vivo* stripping data and the *in vitro* permeation

through the shed snake skin as opposed to the cellulose acetate membrane also lent support to the previous suggestion that the release of diclofenac from the gel base may be the rate-determining step in the overall percutaneous absorption of this drug.

As shown in Table 21, the percent absorbed during the first three hours was not significantly different among the four products. This indicated that the intrinsic absorption of diclofenac through the stratum corneum occurred at about the same rate. Furthermore, the absorption rate was observed to be quite rapid. After three hours, the percent drug absorbed was greater than 70% in all products. On the contrary, data in Table 4 indicated that the percent drug released after six hours, as calculated from the ratio of the cumulative amount found in the receiver compartment to the original amount in the donor compartment (1.5 g of 1.16% gel equivalent to 17.4 mg diclofenac diethylammonium), was in the range of 8.32 to 11.63%. A comparison between the percent *in vitro* drug release at 6 hr and the percent drug percutaneously absorbed at 3 and 6 hr is given in Table 23. The data from this table strongly suggested that the drug release from the vehicle occur at a rate which was much slower than the absorption through the stratum corneum in all the four products.

These findings, together with the results from the correlation test, strongly supported that the difference in the amount of diclofenac initially found in the stratum corneum after termination of the drug treatment was due to the difference in the release characteristcis rather than the absorption rate. As previously described, during the drug application period, two processes occurred simultaneously, namely the release from the vehicle and the penetration of drug through the skin. Since the absorption rate was rapid as

Table 22: Correlation test of *in vitro* and *in vivo studies*

In Vivo Skin stripping	*In vitro* release study		Correlation coefficient	P Value
Amount at 0 hour	Cellulose acetate membrane	release rate	0.9548	p< 0.05
		cumulative amount	0.9326	p >0.05
	Durapore® membrane	release rate	0.8268	p >0.05
		cumulative amount	0.9111	p >0.05
	Shed snake skin	steady state flux	0.3643	p >0.05
		cumulative amount	0.1153	p >0.05
Amount at 1 hour	Cellulose acetate membrane	release rate	0.9878	p<0.05*
		cumulative amount	0.9599	p<0.05*
	Durapore® membrane	release rate	0.8224	p>0.05
		cumulative amount	0.8855	p>0.05
	Shed snake skin	steady state flux	0.5103	p>0.05
		cumulative amount	0.2080	p>0.05
Amount at 3 hour	Cellulose acetate membrane	release rate	0.9649	p<0.05*
		cumulative amount	0.9851	p<0.05*
	Durapore® membrane	release rate	0.7509	p>0.05
		cumulative amount	0.8269	p>0.05
	Shed snake skin	steady state flux	0.5959	p>0.05
		cumulative amount	0.3191	p>0.05
Amount at 6 hour	Cellulose acetate membrane	release rate	0.8458	p>0.05
		cumulative amount	0.9258	p>0.05
	Durapore® membrane	release rate	0.6433	p>0.05
		cumulative amount	0.6851	p>0.05
	Shed snake skin	steady state flux	0.7646	p>0.05
		cumulative amount	0.4488	p>0.05

* = significant at $\propto = 0.05$

compared to the release rate, the difference in the amount found in the stratum corneum immediately after termination of drug application should be a result of the difference in the release profiles. In addition, since the absorption rate was found to be similar among the four products, the significant differences in the amount of drug found in the skin persisted to the later time points as a consequence of the initial difference at time zero (Table 20).

It should be noted that the stratum corneum is generally considered to be the most important barrier against percutaneous absorption (Wu et al,1992). The bioavailability of transdermally administered drugs is largely a function of their permeability across this layer of skin (Bhattachar et al, 1992). Therefore, penetration of the drug through this layer is often the rate-limiting step. This was found to be the case with the shed snake skin. As can be seen from Figures 14-17and Table 8, the *in vitro* cumulative amount penetrated and the steady state permeation flux from this membrane were much lower than the cellulose acetate and Durapore® membranes suggesting its significant barrier properties.

However, results from the skin stripping study in humans were different from the data from the shed snake skin. The results indicated that the human stratum corneum was not the rate-limiting barrier as it was expected. Several explanations could be made on this observation. First, although the shed snake skin is composed of pure stratum corneum, the cellular arrangement of its lipid and protein components is more dense than the human stratum corneum (Itoh et al, 1990). Further, measurements of the thickness of the shed snake skin specimens gave values ranging between 200 - 250 micron (AppendixVI). On the other hand, Barry et al(1983) reported that the thickness

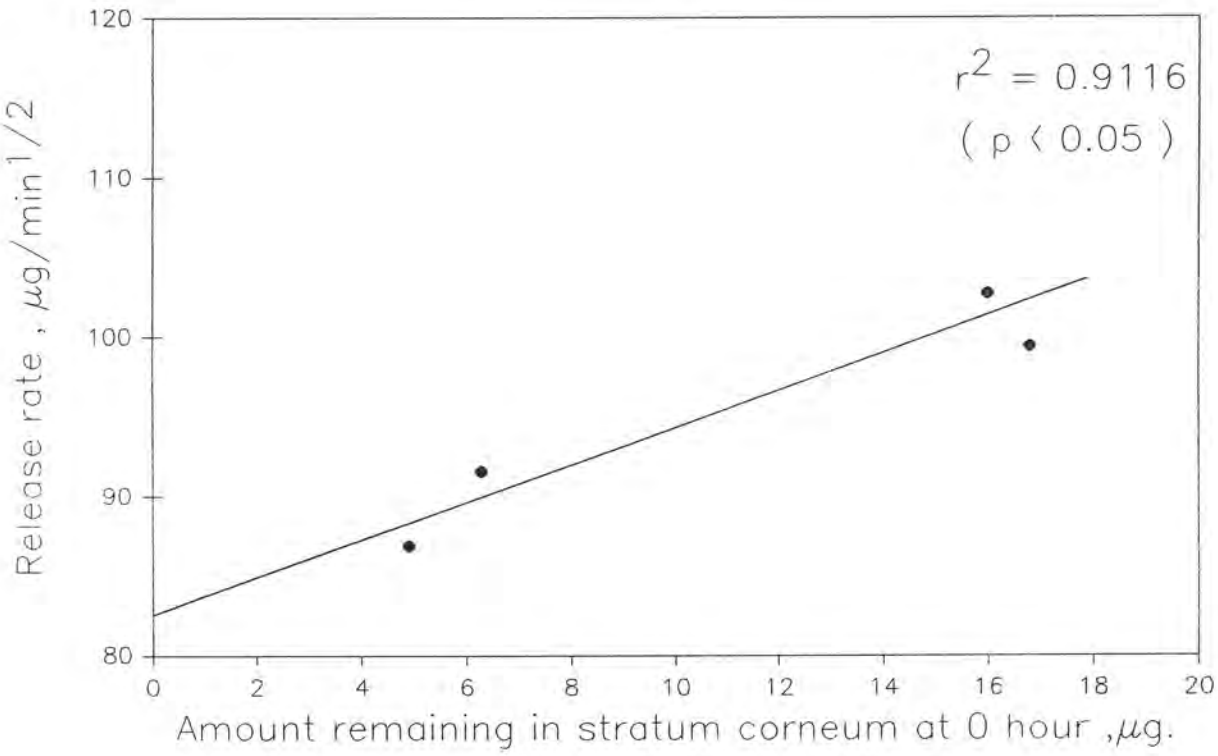

Figure 25: Correlation between the amount of diclofenac in stratum corneum at 0 hour and release rate of diclofenac through cellulose acetate membrane after 6 hour

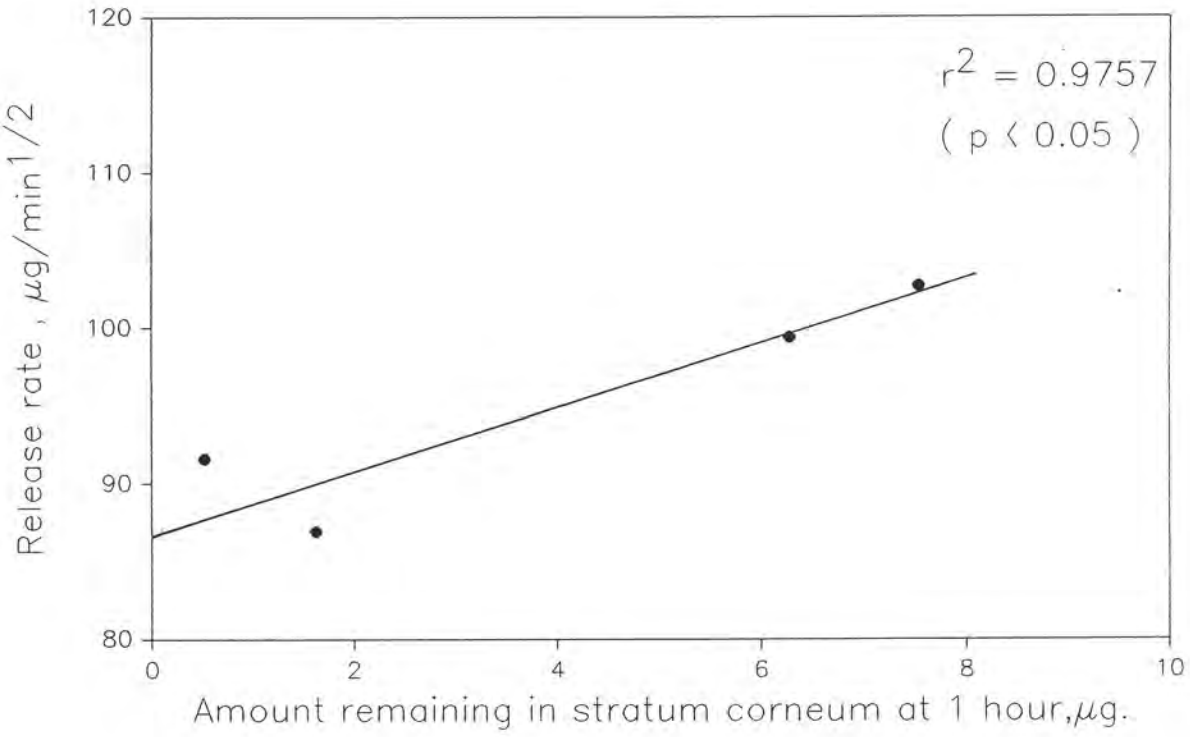

Figure 26: Correlation between the amount of diclofenac in stratum corneum at 1 hour and release rate of diclofenac throug cellulose acetate membrane after 6 hour

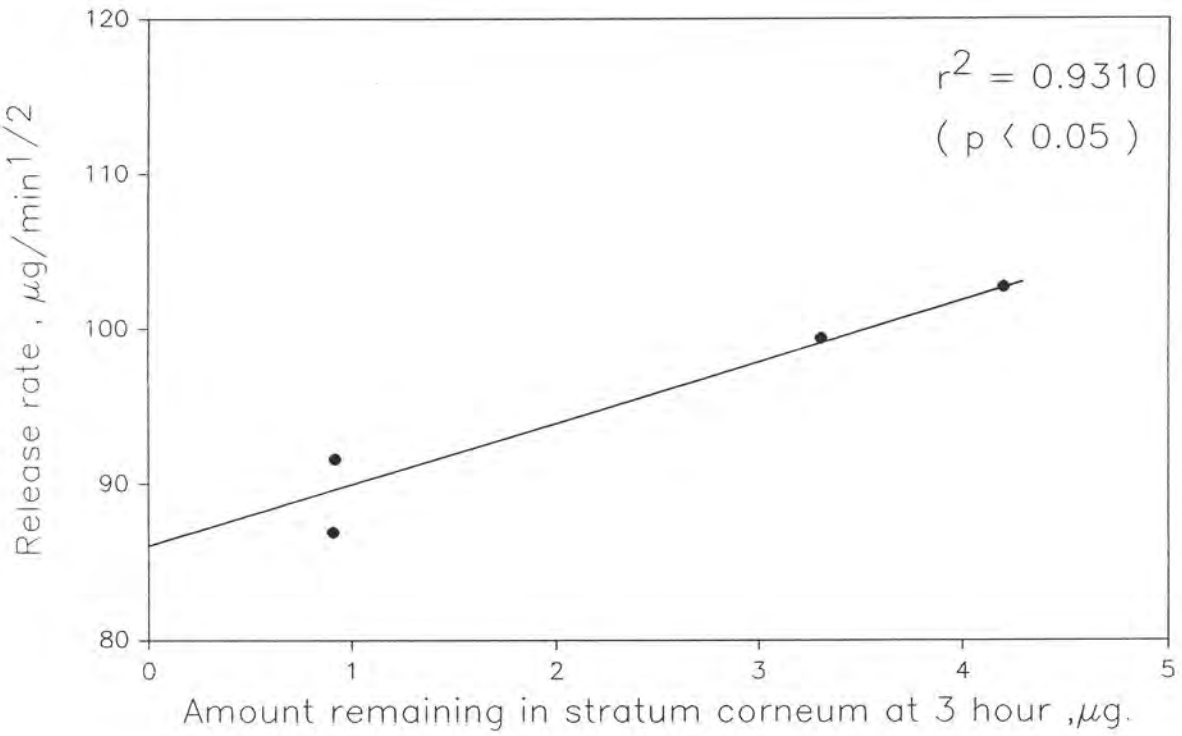

Figure 27: Correlation between the amount of diclofenac in stratum corneum at 3 hour and release rate of diclofenac through cellulose acetate membrane after 6 hour

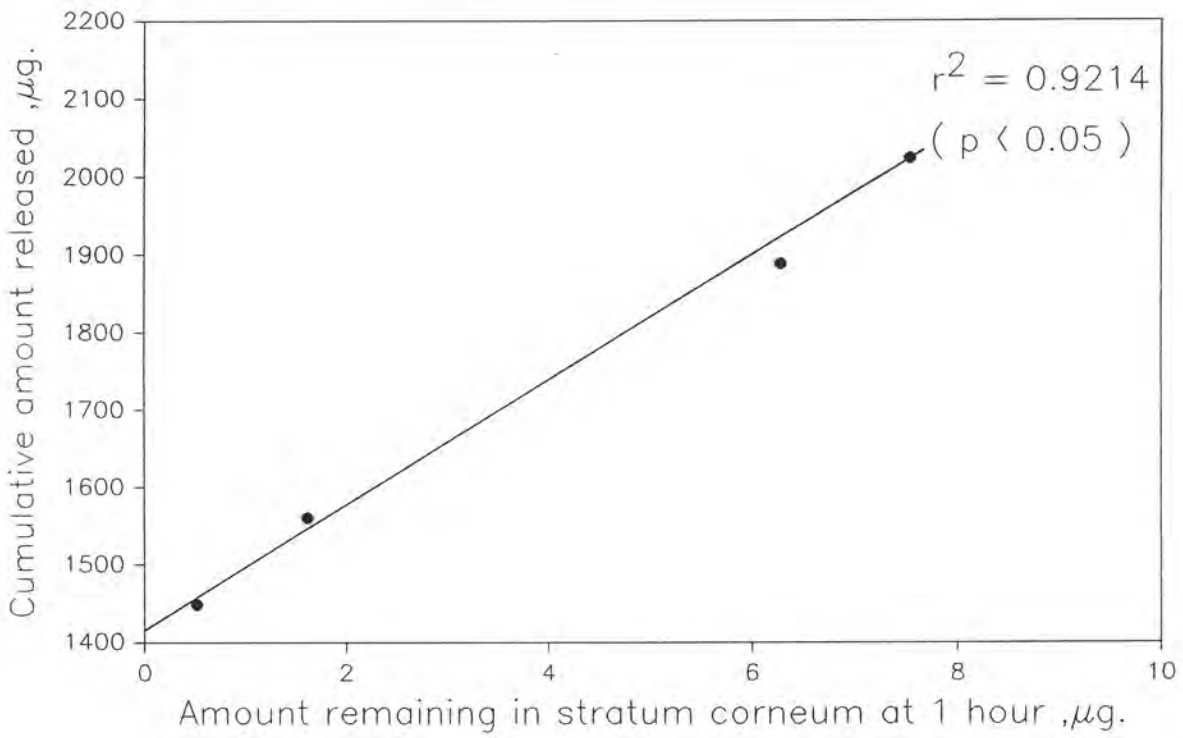

Figure 28: Correlation between the amount of diclofenac in stratum corneum at 1 hour and cumulative amount of drug release though cellulose acetate membrane after 6 hour

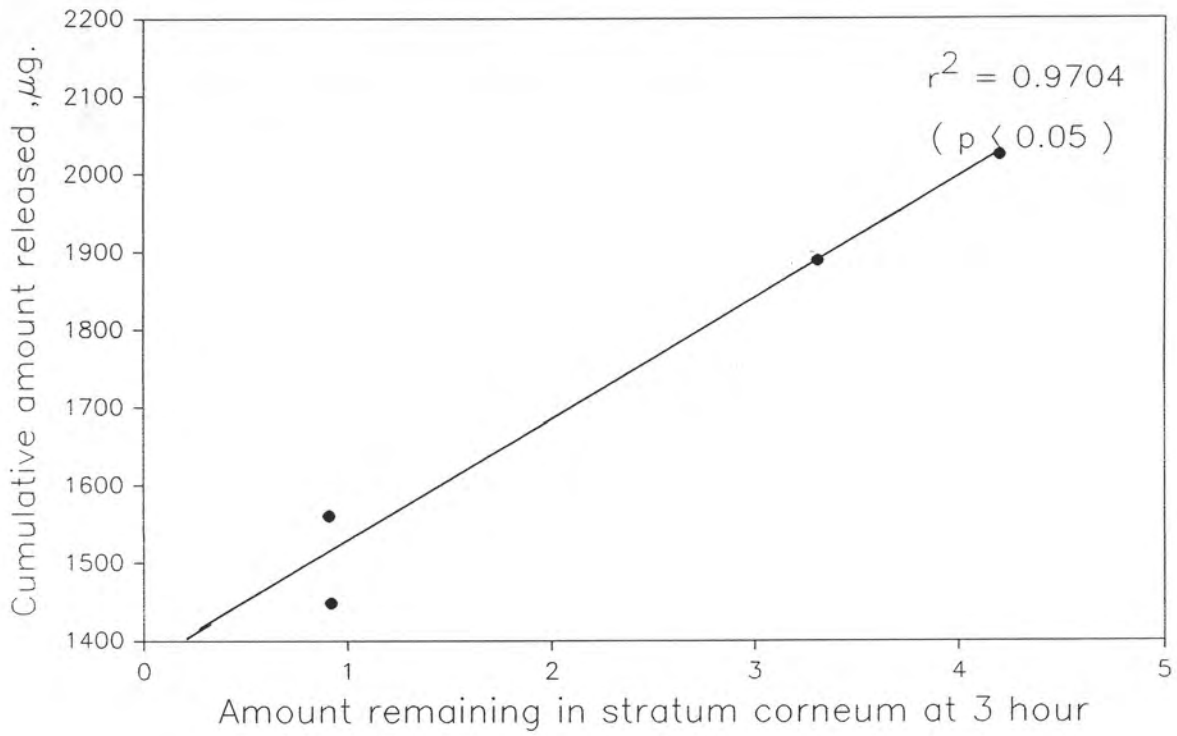

Figure 29: Correlation between the amount of diclofenac in stratum corneum at 3 hour and cumulative amount of drug release through cellulose acetate membrane after 6 hour

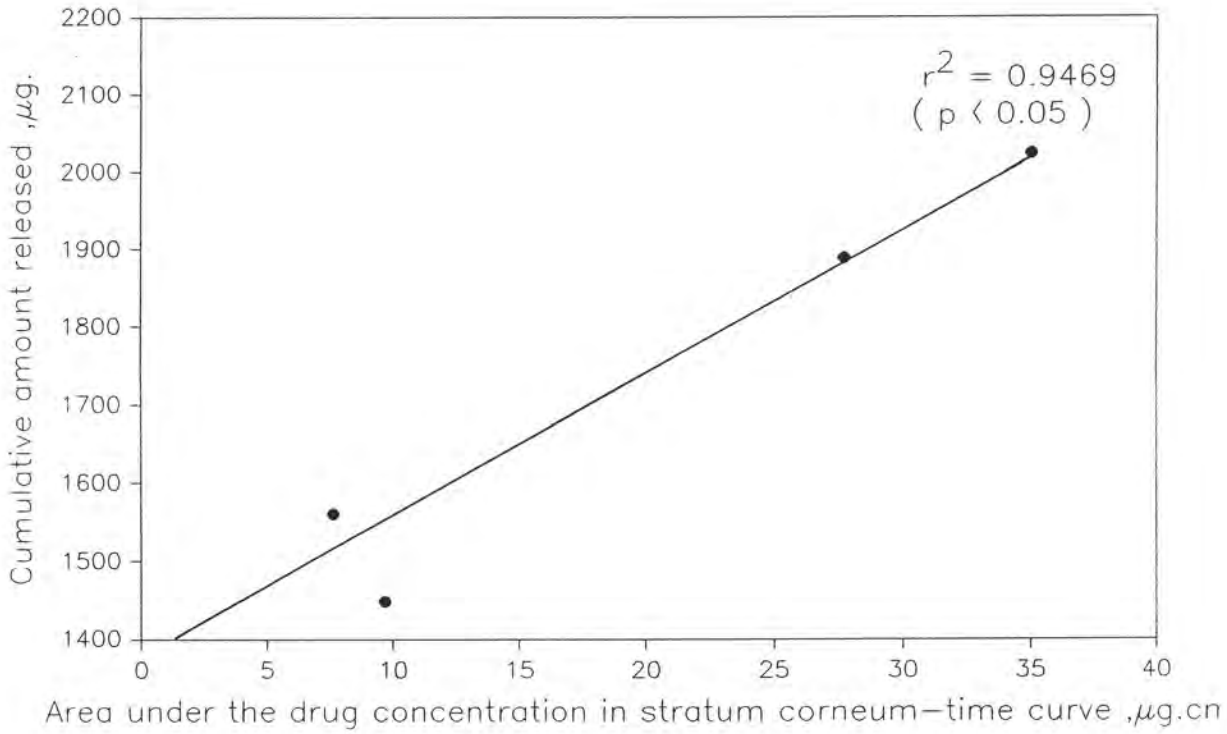

Figure 30: Correlation between the area under the concentration-time curve of diclofenac in the stratum corneum and cumulative amount of drug through cellulose acetate membrane after 6 hour

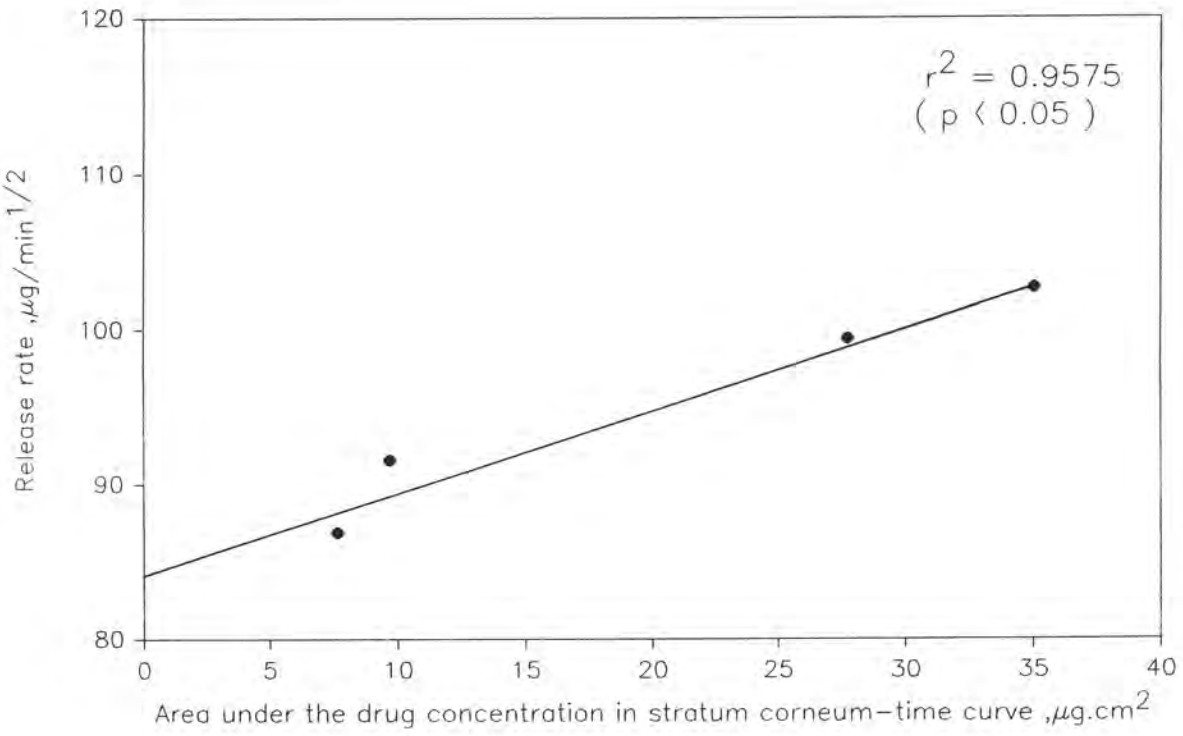

Figure 31: Correlation between the area under the concentration-time curve of diclofenac in the stratum corneum and release rate of diclofenac diethylammonium through cellulose acetate membrane after 6 hour

of the stratum corneum of human forearm is in the range of 10-15 μm the values which are 16 - 20 times smaller than the shed snake skin. Since the permeability coefficient is inversely proportional to the membrane thickness (Feldman, 1967), the permeability of diclofenac through the thin stratum corneum of the human forearm could be much higher than through the shed snake skin.

It should also be pointed out that skin stripping at other areas different from the forearm may give different results since the thickness and number of gland openings of the human stratum corneum varies widely depending on the body site (Feldman,1967). It is possible that application of diclofenac gels to the back of a subject may show percutaneous absorption which may be limited by the penetration rate. Nevertheless, the use of human forearm was fixed thoughout the entire study and comparison of the amount found in this area of the skin can still be used to evaluate diclofenac percutaneous absorption and topical bioavailability among the four preparations.

2.4 Determination of Relative Bioavailability

An attempt has been made in this study to develop a simple technique to evaluate *in vivo* percutaneous absorption in humans. The method was based on the skin stripping approach in which the amount of the drug in the tape-stripped stratum corneum was analyzed following topical administration. Riess et al (1986) reported that the topical application of diclofenac over an inflamed joint of wrists and fingers resulted in synovial fluid

Table 23: Comparison of percent released and average percent percutaneously absorbed of diclofenac in four gel products

Product	*In Vitro* release study	*In Vivo* percutaneously absorbed	
	Average percent released at 6 hour	Average percent absorbed at 3 hr.	Average percent absorbed at 6 hr.
A	10.85	79.39	89.72
B	8.96	77.50	87.12
C	11.63	71.66	78.75
D	8.32	84.91	85.94

drug concentrations which exceeded plasma concentrations. They reported that despite the very low level of diclofenac in plasma after topical administration, significant amount of the drug was still found in the synovial fluids of the joint. These authors thus suggested that diclofenac may have directly penetrated into the joint. However, whether their suggestion was true is still controversial. Radermacher et al (1991) measured the concentrations of diclofenac in the synovial fluids of knee joints, one being treated with the drug and the other with placebo gel. They found that diclofenac was present in both the drug-treated and placebo-treated knees and that the synovial drug concentrations were significantly lower than the plasma drug concentrations. These authors therefore suggested that the direct transport across the skin accounted for only a small fraction of diclofenac reaching the knee joint and that at least 85 % of the dose must penetrate via the systemic circulation. Nevertheless, the measurements of the synovial fluid of the knee joints were taken after four days of topical drug administration. This would allow the drug to penetrate from the treated knee, be systemically absorbed, distributed throughout the body and finally equilibrated into the synovial fluid of the contralateral, placebo-treated knee. Zacher et al (1986) also showed that synovial fluid drug concentrations could be lower than those in the synovial tissues. These findings still indicated that, at least, there was local accumulation of diclofenac in the affected tissues regardless of the route of distribution.

In another study by Seth et al (1991), twelve human subjects participated in the *in vivo* percutaneous absorption experiments comparing the bioavailability of diclofenac from two topical dosage forms, i.e. the solution gel and the emulsion gel preparations. They reported that both products gave absolute bioavailability of less than 10 % when compared to parenteral administration. The peak plasma level was only about 80 ng/ml. These data are in agreement with the results of Seth (1992) who studied the bioavailability of diclofenac from a solution gel and an emulsion gel containing phospholipid

in rats. They reported the bioavailability values, based on the ratio of areas under plasma drug concentration versus time curve (AUC) between the test formulation and the intravenous solution, to be only 5 and 25 % for the solution gel and the emulsion gel, respectively. They also observed a marked accumulation of diclofenac in the tissue in spite of the low bioavailability of diclofenac in plasma. It thus appears that measurement of diclofenac in the target tissues or areas close to the site of action may give better indication of product bioavailability than measurement of drug in the general circulation.

Nishihata et al (1987) studied the *in vivo* percutaneous absorption of diclofenac in rats. They found that there was a good relationship between the plasma diclofenac concentration and diclofenac accumulation in the dorsal skin tissue. In addition, the drug was also accumulated in the subcutaneous tissues underneath the dermal vasculature, suggesting that the diclofenac gel product used in their study could be available for topical treatment rather than for systemic application. As a result, it should be possible to measure the topical bioavailability of diclofenac by means of analyzing the drug concentration in the stratum corneum which has been removed from the drug-treated skin by a series of adhesive tape strips. The amount of the drug found in this layer of skin should reflect the amount that is percutaneously absorbed. The higher amount detected, the greater is the extent of percutaneous absorption and its topical bioavailability.

Since the area under the plasma concentration-time curve is generally used to evaluate the systemic bioavailability of the drug , In the same way, the area under the stratum corneum concentration-time curve could be employed to evaluate the bioavailability of the topical dosage form . Individual data of the area under the stratum corneum conc.-time curve during the 6 hour skin-

stripping (AUC_{0-6}) are shown in Appendix XIV together with the ANOVA and the Duncan's test results . The relative topical bioavailabilities of the four products , as calculated from the ratio of AUC , are in Table 24.

Table 24: *In vivo* topical bioavailability of the three diclofenac diethylammonium gel products (B , C and D) relative to reference product(A)

Product	Relative Bioavailability*
A	100.00 %
B	27.63 %
C	126.41 %
D	34.96 %

* Relative bioavailability = $\frac{\text{AUC(0-6 hr) of sample}}{\text{AUC(0-6 hr) of reference}} \times 100\ \%$ (from amount of drug)

From this data and statistical results in Appendix XIV, it can be seen that only product C was bioequivalent to the reference product A with the relative bioavailability of 126.41 % . Product B and D, on the contrary , were significantly less bioavailability than product A and C with the topical bioavailability of only 27.63 and 34.96 % , respectively. From Appendix XIV ,the Duncan's test result demonstrated that the four products could similarly be divided into two groups, i.e. a group with higher AUC (A and C) and a group with lower AUC (B and D) . Correlation attempt was further made between the AUC and the *in vitro* release parameters. The results are provided in Appendix XIII. Similar to the amount of drug found in the stratum corneum, the AUC_{0-6} also showed significant correlation with both the release rate and

cumulative amount through the cellulose acetate membrane ($p < 0.05$, $r = 0.9731 - 0.9785$).

As a result, the *in vitro* parameters such as the release rate and the cumulative amount released may be used as a rough indicator in predicting the topical bioavailability of diclofenac gel products or to screen for the best formulation during product development.

The application of the *in vivo* skin stripping technique to determine topical bioavailability provides some advantages over the conventional method which normally requires analysis of the drug or its metabolites in the plasma. For example, the analysis of the drug in the tape-stripped stratum corneum is very simple since 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 bioavailability study. The amount of the drug remaining in the stratum corneum can also be measured at various times. Loss of the drug from this layer should confirm the occurrence of percutaneous absorption into deeper skin layers as well as the underlying tissues and systemic circulation.

Due to the simplicity of the design, the technique is thus 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 the 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.

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 known to be systemically absorbed or primarily accumulated in the tissues relatively close to the site of application such as diclofenac, 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.