

CHAPTER III

EXPERIMENTAL METHODOLOGY

Material

Chemical :

Piroxicam, with compliment from Biolab, Co., Ltd., Bangkok, Thailand.

Tenoxicam, with compliment from Biolab, Co., Ltd., Bangkok, Thailand.

Product A = Feldene[®] Lot No. 406-04707 (Mfg.date Dec. 1, 1993), Pfizer International Corp., Bangkok, Thailand.

Product B = Locally made commercial product Lot No. 9483705 (Mfg.date 23/3/94)

Product C = Locally made commercial product Lot No. 067 (Mfg.date 040394)

Product D = Locally made commercial product Lot No. 10401 (Mfg.date 6/7/94)

Product E = Locally made commercial product Lot No. 50XE024 (Mfg.date 270594)

Product F = Locally made commercial product Lot No. VX.61102 (Mfg.date 15/12/36)

Methanol AR grade, Batch No. 3016 KMHB-R, Mallinckrodt Chemical Inc., Kentucky, USA.

Methanol HPLC grade, Batch No. 3041 KLHH, Mallinckrodt
Chemical Inc., Kentucky, USA.

Sodium chloride AR grade, Batch No. 4F027304F, Farmitalia Carlo
Erba, Milano, Italy.

Disodium hydrogen phosphate AR grade, Batch No. 120A578486,
E. Merck, Darmstadt, Germany.

Potassium dihydrogen phosphate AR grade, Batch No. A776073
412, E. Merck, Darmstadt, Germany.

Sodium hydroxide AR grade, Batch No 4L 126055A, Farmitalia
Carlo Erba, Milano, Italy.

Triethanolamine, Viddhayasom, Bangkok, Thailand.

Acetone AR grade, Batch No. 211 K17559306, BDH Laboratories
Supplies, Poole, England.

Histamine, Batch No. 35H5006, Sigma Chemical Co., St. Louis,
USA.

Pontamine Sky Blue 5 BX, Batch No. K21169316450, BDH
Laboratories Supplies, Poole, England.

Oxazolone Batch No. 113H3625, Sigma Chemical Co., St. Louis,
USA.

Membrane :

Cellulose acetate membrane, 0.45 μm pore size, 0.25 mm thickness,
Millipore, Massachusetts, USA.

New born pig skin, kindly donated by pig farm, Nakhonpatom.

Animal : Male albino Swiss mice, supplied by Mahidol University Animal Center, weighing 20-25 g

Male albino Wistar rats, supplied by Mahidol University Animal Center, weighing 108-205 g

Equipment :

- Franz diffusion cells and diffusion apparatus, Crown Glass Company, Somerville, NJ, USA.
- pH Meter, Model 420A, Orion Research Operated, Boston, Massachusetts, USA.
- Analytical Balance, Sartorius 1615 MP, Range 300 gm/1 mg, Werke, Germany.
- Analytical Balance, Sartorius RC 210P, 210 gm, Werke, Germany.
- Thermostatted Circulating Water Bath, Heto InterMed, Heto Birkerod, Denmark.
- Vortex Mixer, Model K-550-GE, Scientific Industries, Inc., Bohemia, New York, USA.
- Ultrasonic Bath, Model 3210, Branson Ultrasonic Corporation, Danbury, Connecticut, USA.
- High Performance Liquid Chromatograph (HPLC) equipped with a tunable UV absorbance detector, Waters 484 Model M 484, Serial No. 484-PRA902, Millipore, Massachusetts, USA.
- an autoinjector, Waters intelligent sample processor, WISP 712, Millipore, Massachusetts, USA.

a constant flow pump, Waters 510 HPLC pump, Millipore, Massachusetts, USA.

an integrator, Waters 745 B Data Modules, Serial No. 7BE/400678, Millipore, Massachusetts, USA.

a spherisorb 5ODS(2), stainless steel column, 250 x 4.60 mm ID, Serial No. 89660, Phenomenex, California, USA.

Method :

Experiments were separated into two parts.

1. *In vitro* evaluation with respect to the percent labeled amount of piroxicam, drug release and membrane permeation characteristics.
2. *In vivo* evaluation
 - 2.1 evaluation of local anti-inflammatory activities using animal models.
 - 2.2 evaluation of *in vivo* percutaneous absorption, using skin stripping technique.

1. In Vitro Evaluation

The first part of the experiments constituted the *in vitro* evaluation of piroxicam topical preparations. The tests included the assay of percent labeled amount and the *in vitro* release/permeation studies using Franz diffusion cells. Six products were selected for this purpose based on their commercial availability and popularity, Feldene[®] (Pfizer) was designated as product A and used as reference product, while products B, C, D, E and F

were locally made commercial products. Their lot numbers and date of manufacture are described in the Material section. All the six products contained the same amount of the active drug, i.e. 0.5%w/w of piroxicam in suitable gel bases.

1.1 Assay of Percent Labeled Amount

Prior to the *in vitro* release/permeation studies, the products were assayed for their contents of piroxicam. The generally accepted assay limit of 90.0 - 110.0 % labeled amount was used as the criteria because the piroxicam gels are unofficial in Pharmacopoeias.

1.1.1 Preparation of Standard Solutions

Standard stock solution was prepared by accurately weighing about 30 mg of standard piroxicam in a 100 ml volumetric flask. Sixty ml of methanol was added and the flask was shaken until the drug was completely dissolved. The solution was then adjusted to volume to make a stock concentration of 0.30 mg/ml with methanol. Ten ml of this solution was pipetted into another 100 ml volumetric flask and adjusted to volume with the mobile phase (described in 1.2.5.5). Then 1, 2, 3, 4, 5, 6 and 7 ml were separately pipetted into 10 ml volumetric flasks and adjusted to volume with the mobile phase. The final concentrations of the standard solutions being 3, 6, 9, 12, 15, 18, 21 $\mu\text{g/ml}$, respectively.

1.1.2 Preparation of Sample Solutions

Approximately 0.5 gm of each of the piroxicam gel products were accurately weighed in a 50 ml volumetric flask. Thirty ml of methanol was added to the flask which was subsequently shaken until the drug was dissolved. The flask was adjusted to volume with methanol. The solution was then filtered through Whatman paper No.1, the first 10 ml being discarded. Each product was prepared in duplicate. 1 ml of the filtrate was pipetted to 10 ml volumetric flask and adjusted to volume with the mobile phase. The sample solutions were then analyzed with HPLC (as described in 1.2.5.5). By comparing the peak area ratio with that of the standard solution, the percent labeled amount of piroxicam in each gel product was calculated.

1.2 In Vitro Release/Permeation Studies

After determining their content, the six products were subjected to the *in vitro* release/permeation tests using Franz diffusion apparatus. The apparatus consisted of six water-jacketed cell holders fixed on the same mounting case with built-in magnetic stirrer. Each cell consisted of two parts, namely the donor and the receiver compartments, vertically attached to each other via a metal clamp. The inside diameter was 1.74 ± 0.11 cm, equivalent to the area of 2.37 ± 0.30 cm². The diagram of the apparatuses is shown in Figure 9. The two compartments were separated by a thin membrane barrier. Two types of membranes were selected for use in this study. These were cellulose acetate membrane and new born pig skin for the *in vitro* release and permeation studies, respectively.

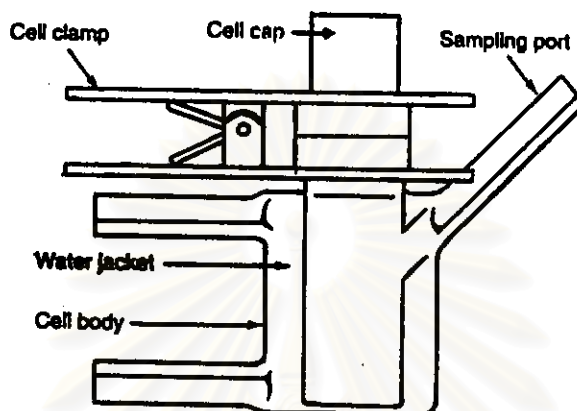


Figure 9 Schematic diagram of the Franz-type diffusion cells

(Courtesy of Crown Glass Company, Inc.)

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Briefly, 1.5 gm of each gel preparation was placed in the donor chamber on top of the receiver compartment which contained pH 7.4, isotonic phosphate buffer solution. The preparation of the buffer is described in 1.2.2. The system was maintained at 37 °C throughout the study by means of a circulating water bath. The steady-state flux and the cumulative amount of piroxicam which was released from the product and penetrated through the membrane were then calculated following the analysis of the drug in the receptor fluid at various times.

1.2.1 Preparation and Pretreatment of Membrane

Cellulose acetate membrane : Cellulose acetate sheets were washed out and rinsed with distilled water before immersing in the pH 7.4, isotonic phosphate buffer for about six hours prior to use. After pretreatment, the membrane sheet was mounted directly onto the diffusion apparatus.

New born pig skin : New born pig skin specimens were selected as representatives of heterogeneous system, the major barrier to percutaneous drug absorption. The abdominal skin of a new born pig was excised and inspected for any defects. Adhering fat and other visceral debris were carefully removed from the inner surface using a pair of scissors. The excised skin was bathed in pH 7.4 phosphate buffer for 1 hour, blotted with clean cloth, wrapped in an aluminum foil, and kept in a freezer. Before using, the frozen skin was thawed at room temperature and then was cut about the same size as the diffusion cell and immersed in pH 7.4, isotonic phosphate buffer for 3 hr prior to use.

1.2.2 Preparation of pH 7.4, Isotonic Phosphate Buffer

1.9 gm of monobasic potassium phosphate was dissolved in 400 ml of distilled water. 4.11 gm of sodium chloride was then added to this solution. 8.1 gm of dibasic sodium phosphate was separately dissolved in another 400 ml of distilled water. The two solutions were then mixed together and the final volume was adjusted to 1000 ml with distilled water. The pH was adjusted to 7.4 ± 0.02 with either 1 M phosphoric acid or 1 N sodium hydroxide solution.

1.2.3 In Vitro Studies through Cellulose Acetate Membrane

The cellulose acetate membrane which had been previously treated was mounted between the donor and the receiver compartments by means of a clamp. The receiver compartment was isotonic phosphate buffer pH 7.4 and was warmed to 37°C with a circulating water jacket which was connected to a constant temperature water bath. The receiving solution was kept well stirred at 300 ± 5 rpm with a magnetic stirrer throughout the time of diffusion studies. The membrane and the receptor solution were equilibrated for 30 min and any air bubbles that had formed under the membrane were removed by gently rocking the cell. All diffusion studies were carried out in triplicate using three separate cells mounted on the same apparatus. After equilibration, an excess amount of piroxicam gel (about 1.5 g) was applied over the membrane surface in the donor chamber of each diffusion cell. Then, the donor chambers were covered with paraffin films and the diffusion of piroxicam were allowed to take place.

At each of the predetermined intervals (30, 60, 90, 120, 180, 210, 240 and 360 min), the receiving solution was sampled via the side arm sampling port of each cell. The entire receiving solution was removed using a syringe fitted with a piece of flexible tubing. The receiving compartment was replaced with fresh pH 7.4, isotonic phosphate buffer, prewarmed at 37 °C and the run was continued. The receiver volumes varied between 12.62 to 13.02 ml depending on the calibrated volume of each diffusion cell.

All the sampled receptor fluids were analyzed using the HPLC technique as described in 1.2.5.5. The piroxicam concentrations in the receptor compartment were determined from the calibration curve. The amount released through the membrane was calculated by multiplying the drug concentration with the respective receptor volume. The cumulative amount of the drug found in the receptor compartment at various time intervals was then plotted as a function of square root of the sampling time. The release rate was subsequently calculated from the slope of the linear portion of each plot. Analysis of variance (ANOVA) was then applied at 5% significance level to see if there were any significant differences in the cumulative amount and the release rate among the six products. If such differences existed, further statistical analysis was performed in order to rank the products using range tests such as Duncan's test.

The six products were then classified into three groups according to their release rate, i.e. fast, intermediate and slow. Then, the three products were selected, each one being representative from each group, for further *in*

vitro permeation and *in vivo* studies, and the results were compared with that of Feldene[®], the standard innovator product.

1.2.4 In Vitro Permeation through New Born Pig Skin

The four piroxicam gel which had been selected from the previous *in vitro* release studies were subjected to the *in vitro* permeation studies using pig skin as a model membrane. The procedure for the permeation through pig skin was similar to that for the drug diffusion through cellulose acetate membrane. However, the pig skin membrane was set in place with the stratum corneum side facing the donor compartment and the dermal side facing the receptor compartment.

The sampling intervals for the pig skin permeation were 3, 6, 9, 12, 15, 18, 21 and 24 hr and the sampled receptor fluids were analyzed using the HPLC technique (described in 1.2.5.5). The cumulative amount of the drug found in the receptor compartment at various time intervals was then plotted as a function of time. The steady-state flux was calculated from the slope of the linear portion of the plot. Analysis of variance (ANOVA) was applied at 5% significance level to see if there are any significant differences in the cumulative amount and the steady-state flux among the four products. Further, statistical analysis was also performed in order to rank the products when significant differences were detected from ANOVA.

1.2.5 HPLC Analysis

The analytical procedure and chromatographic conditions are as follows :

1.2.5.1 Preparation of Tenoxicam Internal Standard Solution

About 20 mg of tenoxicam was accurately weighed in a 100 ml volumetric flask followed by addition of 60 ml methanol. The flask was shaken until the compound was completely dissolved and then adjusted to volume with methanol. 5 ml of the solution was pipetted to another 100 ml volumetric flask and diluted to volume with pH 7.4, phosphate buffer. The final concentration of tenoxicam was 10 $\mu\text{g/ml}$.

1.2.5.2 Preparation of Piroxicam Standard Solutions

About 20 mg of piroxicam was accurately weighed in a 100 ml volumetric flask. Sixty ml of methanol was added to dissolve the drug. After dissolution, the flask was adjusted to volume with the solvent. The concentration of this standard stock solution was 200 $\mu\text{g/ml}$. 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 and 3.0 ml of the standard stock solution were separately pipetted and transferred into 10 ml volumetric flasks. All flasks were subsequently diluted to volume with pH 7.4, phosphate buffer. The final drug concentrations was about 4, 8, 12, 16, 20, 40 and 60 $\mu\text{g/ml}$, respectively. Prior to injection, each standard solution was mixed with equal volume of Tenoxicam internal standard solution.

1.2.5.3 Preparation of Sample Solutions

One ml of the receptor fluid sample was pipetted and similarly mixed with one ml of the internal standard solution. Thus, the concentration of the internal standard prior to the HPLC analysis was kept constant at 5.0 $\mu\text{g/ml}$ in both the standard and the sample solutions.

1.2.5.4 Preparation of 0.085 M Phosphate Buffer, pH 5.6

11.57 gm of potassium dihydrogen phosphate was dissolved in 500 ml distilled water. The pH of the solution was adjusted to 5.6 with dropwise addition of 1 N sodium hydroxide. The final volume was subsequently adjusted to 1000 ml with distilled water.

1.2.5.5 HPLC Conditions for Piroxicam Analysis

The conditions for the analysis can be summarized as follows:

Column	: Spherisorb 5ODS (Phenomenex)
Mobile phase	: A mixture of methanol and pH 5.6, 0.085M phosphate buffer at a ratio of 45 : 55 %v/v (with 1%v/v Triethanolamine in total mobile phase)
Detector wavelength	: 361 nm
Flow rate	: 1 ml/min
Attenuation	: 2-8
Chart speed	: 0.25 cm/min
Injection volume	: 20-40 μl

Internal standard	: 5 µg/ml final concentration of tenoxicam
Retention time	: Piroxicam 8.7 min Tenoxicam 6.6 min

The representative chromatograms of the standard and the sample solutions, together with the standard curve are shown in Figure 10.

2. In Vivo Evaluation

2.1 In Vivo Evaluation of Local Anti-inflammatory Activities

After the *in vitro* tests, the four gel products were subjected to the *in vivo* evaluation. The test comprised the assessment of the local anti-inflammatory activities of topical preparations after application in two animal models to provide supportive evidence of their effective percutaneous absorption.

Topical application of nonsteroid anti-inflammatory drugs (NSAIDs) 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. 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.

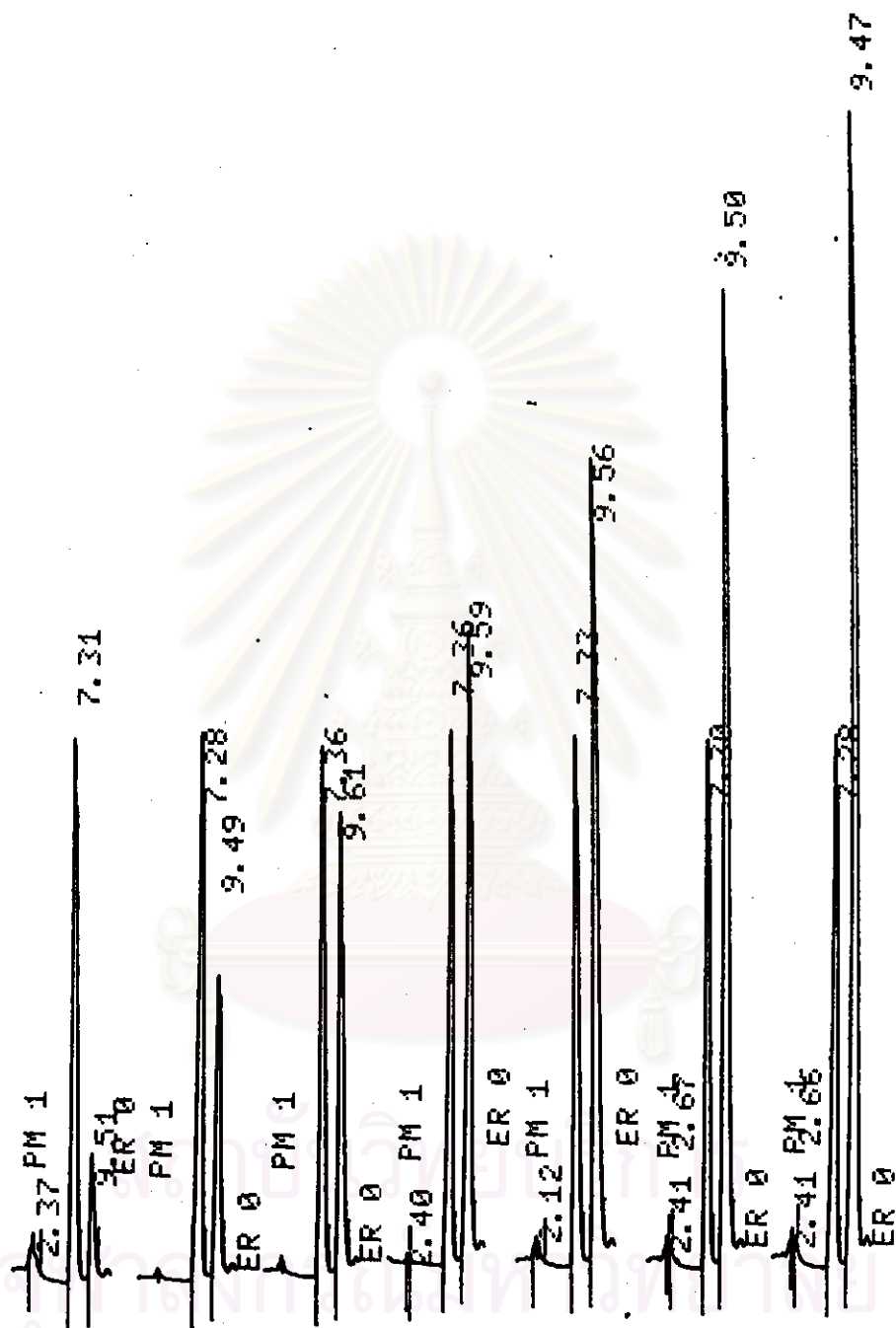


Figure 10 Chromatograms and standard curve of piroxicam at concentrations: 3.21 (1), 6.42 (2), 9.63 (3), 12.84 (4), 16.05 (5), 19.26 (6) and 22.47 (7) $\mu\text{g/ml}$.

(A = Piroxicam, B = Internal standard or Tenoxicam 8.36 $\mu\text{g/ml}$)

Recent studies to produce models of allergic contact hypersensitivity have been successful, both from the standpoint of the lesion induction and the 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. This mouse model has been used to investigate the immunosuppressive properties of certain anti-inflammatory drugs (Bouclier et al, 1989).

Another study, passive cutaneous anaphylaxis (PCA) can be elicited by an intravenous injection of antigen to animals previously sensitized intracutaneously with homologous or heterologous antibodies. The measure of PCA reaction is generally quantified using radioactive isotopes and Evan's blue dye as indicators of capillary permeability. PCA has been of great value in *in vivo* studies on immediate hypersensitivity reactions (Bouclier et al, 1989).

2.1.1 Oxazolone-induced Ear Edema

The four gel products which have been tested for their *in vitro* permeation, were then subjected to the *in vivo* evaluation of their anti-inflammatory activities by monitoring the ability to suppress or prevent local edema. The technique was based on the oxazolone-induced delayed hypersensitivity assay as described by Evans et al. (1971) with some modifications.

In this test oxazolone (4-ethoxymethylene-5-oxo-2-phenyl-4,5-dehydrooxazole) was used to induced hypersensitivity reaction in the mouse ears. Oxazolone is a potent sensitizing agent which can rapidly induce edema and erythema upon contact with the skin. Sixty male albino Swiss mice, weighing 20-25 g, were obtained from Mahidol University Animal Center. The animals were sensitized by applying 100 μ l of the freshly prepared oxazolone solution (2% in acetone) to the unshaved abdomen on days 1 and 2 (Figure 11) using a needle connected to a 1 ml syringe. On day 5 at 8.00 a.m. each animal was challenged by direct application of the oxazolone solution to the right ear, the solution being divided into two aliquots of 50 μ l each for thorough application to both sides of the ear. One hour later, about 70 mg of each of the four products was applied to the previously sensitized right ear using small spatula. Care was taken during application to ensure that the right ear was completely covered with the product on both sides. The drug application was repeated two more times at 3 and 6 hr after oxazolone challenge. This was to make sure that the applied drug was in excess during the initial period of sensitization. The repeated application also resembled the actual drug administration whereby the product should be applied as early and frequently as possible following the onset of skin inflammation. The left ear was not challenged nor treated with the product so as to serve as the self control within each animal. Twenty-four hr after challenge (day 6 at 8.00 a.m.), the animals were sacrificed with chloroform vapor. Both the left and right ears were removed. Each ear tip was then punched out to form a circular piece of 7 mm diameter. Each piece was then weighed to the nearest 0.1 mg using an analytical balance.



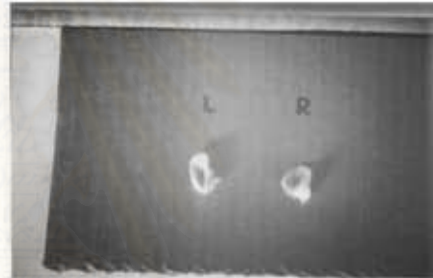
a. Each mice was sensitized by oxazolone solution to the unshaved abdomen.



d. Each mice was sacrificed with chloroform vapor.



b. Each mice was challenged by oxazolone solution to the right ear.



e. Both left and right ears were removed.



c. The gel was applied to the previously sensitized right ear using small spatula.



f. Each ear was punched out to form a circular piece of 7 mm diameter.

Figure 11 Experimental step for evaluation of local anti-inflammatory activity using oxazolone-induced ear edema

The difference in the weight between the right (treated) and the left (untreated) ears was calculated for each mouse. A group of twelve animals was used for each of the four products. In addition, another group of twelve mice was sensitized and challenged with oxazolone but no product was applied to the right ear. This group of animals, therefore, served as a control group. By comparing the difference in ear weight among various groups using the analysis of variance (ANOVA) and multiple range test, implications could be made regarding the effectiveness of these preparations in suppressing the oxazolone induced ear edema.

2.2 Changes in Vascular Permeability Studies

Vascular permeability was measured as described by Hiramatsu et al. (1990). This model employed fifty male albino Wistar rats, supplied by Mahidol University Animal Center, weighing within a range of 108-205 g. By dividing the rats into 5 groups of 10 for control and four products, the test is as follows :

The day before testing, the large area on the back of each rat was shorn with an electric hair clipper and scissors until the skin was hairless (Figure 12). Then 24 hr next, each 100 mg gel was massaged into three circular sites (2 cm diameter each), along the midline with the rubber-gloved finger. One hour later 100 mg gel was again applied on each site. After a further hour the application sites were washed with warm water in order to clean the skin. The control group was not applied with any drug for comparing with the piroxicam gel products. Then all groups were treated



a. The back of each rat was shorn and marked 3 circular sites ($\varnothing = 2$ cm).



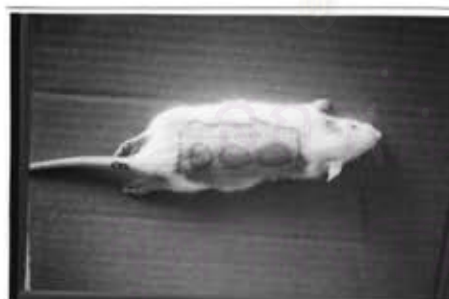
d. The center of each application sites was injected intradermally by histamine solution.



b. The gel was massaged into the application sites.



e. Injected histamine intradermally into 3 circular sites.



c. The gel was massaged into three circular sites.



f. Pontamine sky blue solution was injected intravenously into the tail vein.

Figure 12 Experimental step for evaluation of local anti-inflammatory activity using histamine-induced vascular permeability



g. The rat was decapitated.



h. The treated skin was removed.



i. The size of the blue spots was measured.

Figure 12 Experimental step for evaluation of local anti-inflammatory activity using histamine-induced vascular permeability (continue)

with 100 µg histamine dissolved in 0.1 ml water (concentration 1 mg/ml) by intradermal injection into the center of each application site. Immediately thereafter, 2 ml/kg of a 2% solution of pontamine sky blue was injected intravenously into the tail vein. Twenty minutes later, all animals were decapitated and the treated skin removed. The size of the blue spots was determined by multiplying the shortest by the longest diameter and expressed in mm² per animal as the average of the three sites. So, the greater the area of the blue spot, the less the inhibition of vascular permeability of the product.

The areas of the blue spots were then compared among the five groups using one way ANOVA and an appropriate rank test at 5% significance level.

2.2 Evaluation of Percutaneous Absorption of Topical Piroxicam Gel Products Using *in Vivo* Skin Stripping Technique

Tengamnuy et al. (1996) previously have applied this technique to quantitatively assess the topical bioavailability of diclofenac diethylammonium gels. They also found a correlation between the release rate through the cellulose acetate membrane and the amount of diclofenac found in the stratum corneum at all time points during the first three hours.

Following application of the product to the designated area i.e. the mid-forearm, the stratum corneum was sequentially removed from the skin surface by means of adhesive tape strips. Each skin stripping with a piece of

adhesive tape resulted in a certain amount of stratum corneum being removed. Analysis of drug in the tape-stripped stratum corneum would reveal the amount of drug that was released and permeated through the upper layers of the skin. Topical bioavailability was thus estimated from the amount of drug found in the tape stripped stratum corneum following specific period of drug application.

The greater the initial amount of drug found after administration, the higher the extent of drug release and penetration. This would therefore reflect the higher degree of percutaneous absorption and topical bioavailability.

This part of study attempted to apply the skin stripping technique to another NSAIDs such as piroxicam. The experimental conditions followed those by Tengamnuay et al. (1996), particularly regarding the appropriate skin stripping sequence and the optimum occlusion time after drug application.

The four piroxicam gel products were subjected to the *in vivo* skin stripping test. The study was a single-blinded, completely randomized crossover design employing eight healthy volunteers. Four females and four males, with age ranging from 22 to 30 years (average 26), participated in the study. All subjects had not taken any oral and/or topical medications for at least one week before and during the study.

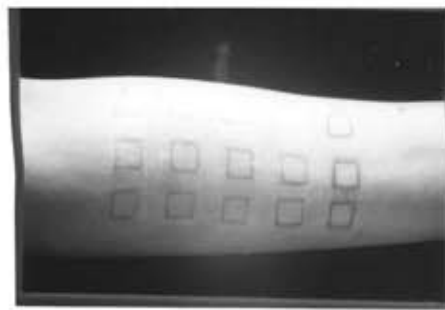
Each volunteer received a completely randomized sequence of the four products on four occasions, separated by a washout period of two weeks. By means of a paper template with twelve punched holes, the area of their left mid-forearms was marked by pen into corresponding twelve small squares, each having a $1 \times 1 \text{ cm}^2$ dimension (Figure 13). These squares were called the application spots on which the product was to be applied. There were four parallel rows of spots, each row consisting of three spots in a 4×3 arrangement. The position of the spots in the forearm was such that the top row was at least 3 cm below the antecubital fossa and the lowest (fourth) row about 3 cm above the wrist, each row being separated by a distance of 1.0 cm.

The same excessive dose (about 100 mg) of each product was subsequently applied on each of the twelve spots using a small spatula. Care was taken to make sure that the same amount of the gel was applied to each spot and that the gel contained within the spot boundary. Immediately after drug administration, each row was covered with Tegaderm[®] tapes for 3 hr (Samitamam, 1995). After this occlusion period, Tegaderm[®] tape were removed and any residual gel was gently but thoroughly wiped out three times from the surface of each spot using cotton buds.

One row of spots was then randomly selected for skin stripping at time zero whereas the remaining three rows were covered again with new pieces of Tegaderm[®]. The stripping procedure was then started, using ten pieces (or strips) of Transpore[®] tape, cut to about the same size as the



a. The area was marked by pen
(each of spot was 1 cm^2).



e. At appropriate time (1, 3 and 6 hr), the covering were removed.



b. The application spots were 4 x 3 arrangement.



f. Residual gel was wiped out by cotton buds.



c. The excessive dose of gel was smeared on each spot.



g. Ten strips of Transpore[®] were placed, steady pressure was applied by a small roller.



d. Occluded with Tegaderm[®].



h. Transpore[®] tape was removed with a small forcep.

Figure 13 Experimental step for evaluation of percutaneous absorption using skin-stripping technique

application spots ($1 \times 1 \text{ cm}^2$). Each Transpore[®] tape strip was consecutively placed on an application spot by means of small forceps. After placing the first piece, a steady pressure was applied to the application spot using a small roller before removing the tape away from the skin surface. The second stripping was performed in the same manner. However, discarding the first two strips as Samitamarn's studies had shown that they contained the excess amount of the residual, unabsorbed gel. The remaining strips were repeated the same procedure and combined together for each spot. Each piece of tape was used to strip the skin only once. Following the first application spot, the second and the third spot in this row were repeated in the step of procedure. All strips (24 strips) were weighed together by analytical balance (Sartorius RC 210P) before and after stripping procedure to obtain the weight of the removed stratum corneum at time zero and kept in a $13 \times 100 \text{ mm}$ culture tube.

The amount of piroxicam found in this row was designated as the initial amount detected in the stratum corneum at time zero after termination of the drug administration. Another row was then randomly selected for stripping when one more hour had elapsed to determine the amount of drug remaining at one hour after termination of drug administration. Similarly, the remaining two rows were randomly stripped at 3 and 6 hr after termination of the drug administration to determine the amount of drug remaining in the stratum corneum after 3 and 6 hr, respectively.

The combined 24 strips from each row were immersed in 3 ml of the mobile phase. After vortexing for one minute, the mixture was centrifuged at 3000 rpm for 5 min, 1.0 ml of the clear supernatant was added to 1.0 ml of 10 µg/ml tenoxicam as the internal standard and then injected onto the HPLC. Details of the analytical condition were similar to the *in vitro* tests as described in 1.2.5.5.

The study would be terminated immediately if major pruritus, redness, or burning sensation were observed at the site of application as a result of the allergic reactions to the drug or to the gel components. After HPLC analysis, the amount of piroxicam found in the tape-stripped stratum corneum was divided by the weight of the stratum corneum removed and then plotted as a function of time. The percent of drug percutaneously absorbed at various times as well as the first order penetration rate constants were also calculated for each product for each subject and analyzed using randomized block ANOVA and an appropriate rank test at 5% significance level (e.g. Duncan's test). Statistical comparison was also made with respect to the amount of drug found in the stratum corneum at different times between the four products. In addition, the area under the concentration-time curve of all products were calculated and used in comparison of the relative bioavailability between the local products and the reference product.