MATERIALS AND METHODS

TEST COMPOUND

PNQ-4482, the pyranonaphthoquinone compound isolated from the dead heart wood of *Ventilago harmandiana* Pierre was obtained from Prof. Dr. Vichai Reutrakul, the Department of Chemistry, Faculty of Science, Mahidol University.

EXPERIMENTAL ANIMALS

Male and female Sprague-Dawley rats weighing 40-60 g, 100-120 g, 140-180 g 150-200 g and 200-250 g as well as male Swiss albino mice weighing 20-40 g were purchased from the National Laboratory Animal Center, Nakorn Pathom. All animals were kept in the room maintained under environmentally controlled conditions of 24 $\pm$ 1° C and 12 h light-12 h dark cycle. The animals had free access to water and standard diet. They were acclimatized at least one week before starting the experiments.

PREPARATION OF TEST DRUGS

All test drugs were suspended in 5% Tween 80, except in the ear edema model, where they were dissolved in acetone.

DRUG ADMINISTRATION

All test drugs were orally administered in an equivalent volume of 0.5 ml/100 g body weight of the animal except in the writhing response model, test drugs were administered intraperitoneally; and in the ear edema model, a local application of test drug to outer and inner surfaces of the ear was performed. Control groups received vehicle only in same the volume and the same routes.

EXPERIMENTAL PROTOCOL

1. Anti-inflammatory study

1.1 Ethyl phenylpropiolate (EPP) and arachidonic acid (AA)-induced ear edema in rats (Brattsand *et al.*, 1982; Yong *et al.*, 1984)

Male rats weighing 40-60 g were used. EPP and AA were dissolved in acetone at the concentration of 50 mg/ml. Ear edema was induced by the topical application of either EPP or AA to the inner and outer surfaces of both ears by means of an automatic microliter pipet. Each rat received EPP 1 mg/20 µl/ear or AA 1 mg/20 µl/ear.

PNQ-4482 as well as reference drugs, phenylbutazone and phenidone, were dissolved in acetone and applied topically in a volume of 20 µl to the inner and outer surfaces of the ear just before the irritants. The control group received acetone. Before and at 15, 30, 60 and 120 min after edema induction, the thickness of each ear was measured with vernier calipers. The increase in the ear thickness was compared with the vehicle-treated group and the percent inhibition was calculated as follows:

$$ED_x = ET_x - ET_o$$

$$\%ED = \frac{ED_c - ED_t}{ED_c} \times 100$$

where,

ED_x = edema thickness at time x

ET_x = ear thickness (µm) at time x

ET_o = ear thickness (µm) before application of EPP or AA

ED_c = edema thickness (µm) of control group at time x

ED_t = edema thickness (µm) of test group at time x

% ED = percent edema inhibition of test compound at time x

1.2 Carrageenin-induced hind paw edema in rats (Winter *et al.*, 1962)

This method was used for investigation of the inhibitory effect of anti-inflammatory drugs on the edema formation of the rat paw induced by carrageenin.

Male rats of 100-120 g body weight were divided into groups of 6 animals. PNQ-4482 and aspirin were orally given 1 h prior to carrageenin injection. The control group received 5% Tween 80 only. Lambda carrageenin was made up as a 1% suspension in sterile normal saline solution. A volume of 0.05 ml of 1% carrageenin was injected intradermally into the plantar side of the right hind paw of an unanesthetized rat, which was restrained in a plastic cage.

Foot volume of animal was determined by means of a volume displacement technique using a plethysmometer (model 7150, Ugo Basile, Italy). The right hind paw was immersed into the measuring chamber containing 0.05% NaCl in distilled water, exactly to an ink mark at anatomical hair line. Each paw volume was obtained from the average of 3 readings. The paw volume was measured prior to and 1, 3 and 5 h after carrageenin injection. The scheme of experiment is shown in Figure 3.

The edema volume of the paw and the percent edema inhibition of each test compound were obtained by the following calculation:

$$EV_x = PV_x - PV_o$$

$$\%EI_x = \frac{EV_x \text{ of control group} - EV_x \text{ of test group}}{EV_x \text{ of control group}} \times 100$$

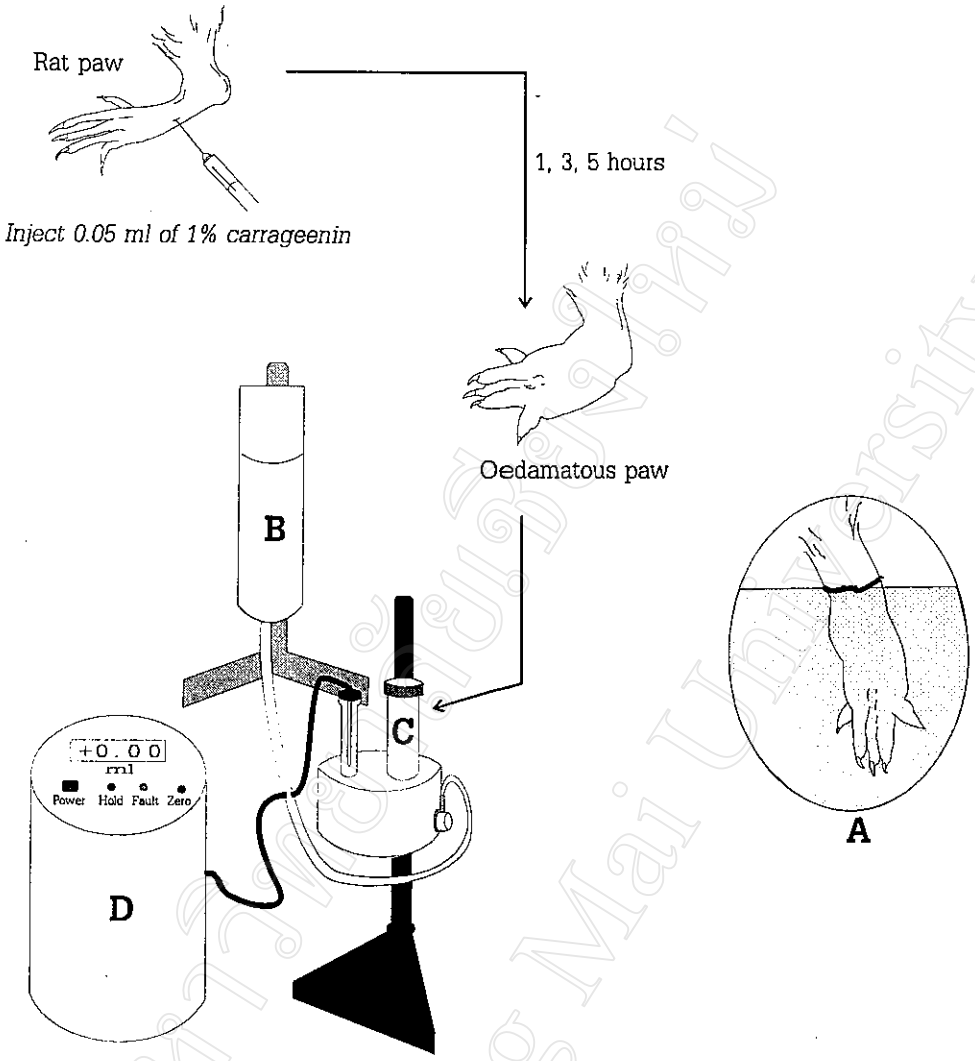
where,

EV_x = edema volume (ml) at time x

PV_x = paw volume (ml) at time x

PV_o = paw volume (ml) measured before carrageenin injection

$\% EI_x$ = percent edema inhibition of test compound at time x



A : measured paw with line indicated anatomical hair line marked

B : water reservoir contained 0.05% NaCl in distilled water

C : measuring chamber

D : plethysmometer

Figure 3. Diagram illustrating the water displacement method for measuring rat paw volume

1.3 Carrageenin-induced pleurisy in rats (Capasso *et al., 1975*)

The model was adopted to investigate the activity of agent on acute phase of inflammation and to study some mechanisms of action.

Male rats weighing 200-250 g were used. A 1% carrageenin in sterile normal solution was injected into the right pleural space of animals under light ether anesthesia in a volume of 0.15 ml. Each intrapleural injection was given between the third and the fifth rib on the right side of the mediastinum after sterilization with 0.5% tincture hibitane. PNQ-4482, aspirin, prednisolone and control vehicle were given orally 1 hour before carrageenin injection. The scheme of experiment is shown in Figure 4.

To determine the anti-inflammatory activity and the mechanisms of action of such compounds, the following experiments were performed.

1.3.1 Exudate collection and volume determination (Vinegar, *et al.,* 1973)

Each animal was killed by a large dose of pentobarbital 3 h after carrageenin injection. The chest was carefully opened and a 5 ml plastic syringe with 16-gauge intubation needle was used to aspirate the fluid from both sides of the thoracic cavity. The exudate was transferred to a 15 ml conical centrifuge tube and the total volume was determined.

1.3.2 Leukocyte determination (Brown, 1980)

The exudates were drawn up by using the white cell pipet to the 0.5 ml mark and diluted with 3% acetic acid by slowly aspirating into the pipet until the mixture reached the 11 ml mark. After shaking well, the first few drops were discarded and the rest was then transferred into a hemocytometer (Neubaner, Boe Co., West Germany). The total leukocyte count was determined by using a microscope (Olympus, Japan)

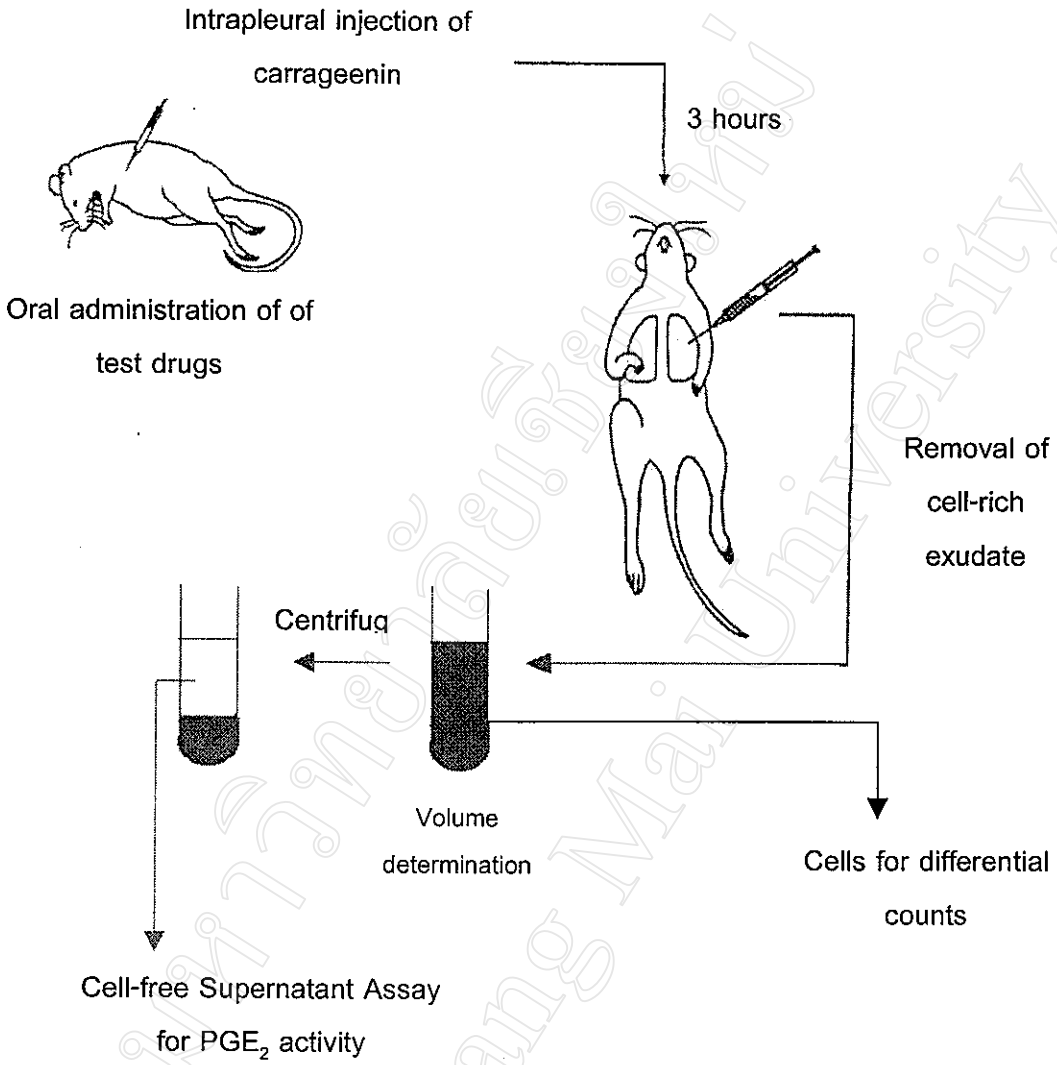


Figure 4. Diagram illustrating the method for carrageenin-induced pleurisy in rats

1.3.3 Prostaglandin extraction (Velo, *et al.*, 1973)

The exudates collected from rats in each experimental group were pooled together and an excess volume of absolute ethanol was added in order to precipitate the protein and the cell material. The mixture was centrifuged at 1000 r.p.m. for 20 minutes. The supernatant was then dried up at 45°C using a vacuum rotary evaporator (EYELA, Tokyo, Rikaikai Co., Ltd., Japan). The dried residue was then dissolved in 4 ml of distilled water and acidified with 1.0 N hydrochloric acid to pH 3. Prostaglandins were extracted from this solution three times with an equivalent volume of ethyl acetate. The combined extract was then evaporated to dryness at 55°C. The residue obtained was stored in a refigerator (0-4°C) while awaiting for an assay, but not longer than 4 days before bioassay was performed.

1.3.4 Bioassay for prostaglandin activity using rat fundus strip preparation (Vane, 1957)

Rats of both sexes, weighing 200-250 g were used. The animals were fasted over-night before the experiment. Each animal was killed by a blow on the head and the abdomen was opened. The fundus part of the stomach was dissected out and strips made following the method described by Vane (1957). The strip was then mounted in a tissue chamber containing oxygenated Tyrode's solution with controlled temperature at 37°C. The Tyrode's solution contained, in addition, the following specific antagonists: chlorpheniramine maleate (10^{-7} g/ml), methysergide hydrogenmaleate ($2x10^{-7}$ g/ml) and atropine sulfate (10^{-7} g/ml). After 45 minute-equilibration, the fundus strip was set to the resting tension of 1.0 g. The isometric contraction was measured via a force displacement transducer (Model FT 03C, Grass Instrument Co., Quincy, Mass., U.S.A.) (Figure 5). After each challenge, the strip was washed three times and allowed to recover for 4 - 5 minute before the next trial. The prostaglandin residue was dissolved in 1 ml of Tyrode's solution. The standard stock solution of prostaglandin E_2 (PGE_2) was also diluted with Tyrode's solution before use. Prostaglandin was assayed by bracketing the contraction induced by injection of the residue exudates between the

smaller and the larger contraction induced by standard PGE_2 (Staff of the Department of Pharmacology, University of Edinburgh, 1970). The result was expressed in term of PGE-like activity according to the following formula.

The amount of standard PGE_2, which should produce the same response as the dose of extracted prostaglandin

$$= S_1 \text{ antilog} \frac{T - S_1}{S_2 - S_1} \left(\log \frac{s_2}{s_1} \right)$$

Where

s_1 = the smaller dose of standard PGE_2 (ml)

s_2 = the larger dose of standard PGE_2 (ml)

T = mean effect produced by extracted prostaglandin (mm)

S_1 = mean effect produced by s_1 (mm)

S_2 = mean effect produced by s_2 (mm)

1.4 Cotton pellet-induced granuloma formation in rats (Swingle and Shideman, 1972)

This experiment was performed for investigation of the ability of an agent to inhibit the proliferative component of the subchronic and chronic inflammatory process. The method was slighly modified as follows:

Adsorbent cotton wool was cut into pieces weighing 20 ± 1 mg and made up to a pellet. The pellets were then sterilized in a hot air oven (model 25, Arthur H. Thomas CO., U.S.A) at 120°C for 2 h.

Male rats of 180-200 g body weight were used. Two pellets were implanted subcutaneously, one on each side of the abdomen of the animal under light ether anesthesia and sterile technique (Figure 6). The suture was then made and the animal was allowed to recover.

PNQ-4482 and prednisolone as well as aspirin were administered orally in a once daily dosage regimen throughout the experimental period of 7 days whereas the

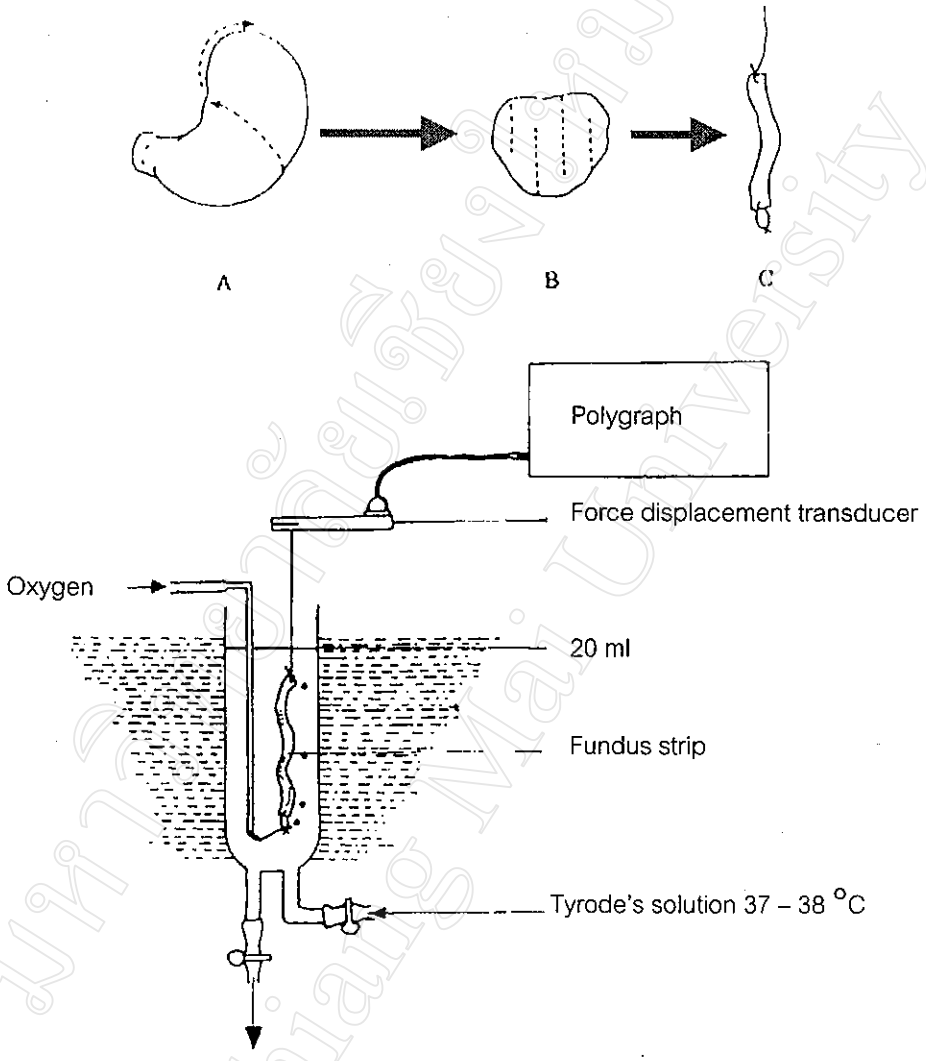


Figure 5. Experimental set for bioassay of prostaglandin by using rat fundus strip preparattion

A : intact stomach with line of incision indicate

B : fundus part, line indicated the desired incision

C : threads tied to each end of the fundus strip

control group received 5% Tween 80 only. The animals were sacrificed on the eighth day after implantation. In order to obtain the data of many parameters, rats were first anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally). The trachea was cannulated with a polyethylene tube to facilitate spontaneous respiration and thereby increase the heart function. The cannula was inserted into the common carotid artery and blood was collected into a tube. The blood was centrifuged at 1000 r.p.m. for 5 min and the serum was separated.

After collection of the blood the rat was sacrificed and the abdominal skin was then opened. The implanted pellets were dissected out and carefully removed from the surrounding tissues and weighed immediately for the wet weight. The thymus was also dissected out. Both cotton pellets and thymuses were dried at 60° C for 18 h and their dry weight determined. The change in body weight of the animals from the first and the last day of experiment was also recorded.

The granuloma formation, transudative weight and the percent granuloma inhibition of the test compound were calculated according to the following formulae:

$$\text{Transudative weight} = Wt_w - Wt_d$$

$$\%GI = \frac{\text{GW of control group} - \text{GW of test group}}{\text{GW of control group}} \times 100$$

$$\text{GW (mg/mg cotton)} = \frac{Wt_d - Wt_i}{Wt_i}$$

where,

Wt_w = wet weight of granuloma pellet (mg)

Wt_d = dry weight of granuloma pellet (mg)

Wt_i = initial dry weight of cotton pellet determined before implantation (mg)

GW = granuloma weight (mg)

%GI = percent granuloma inhibition

Samples of serum were sent to Central Laboratory, Faculty of Medical Technology, Chiang Mai University, for determination of the amount of alkaline phosphatase and total protein. The enzyme activity was expressed as units of enzyme/mg of serum protein.

The method for measurement of alkaline phosphatase activity and total protein in the serum (Bessey *et al,*. 1946) was briefly described as follows: Alkaline phosphatase activity was measured by the kinetic rate method using a 2-amino-2-methyl-1-propanol (AMP) buffer. In the reaction, alkaline phosphatase catalyzed the hydrolysis of the colorless organic phosphate ester substrate, p-nitrophenylphosphate, to the yellow colored product, p-nitrophenol, and phosphate. This reaction occurred at an alkaline pH of 10.3. Total protein concentration was measured by a timed-endpoint biuret method. In this reaction, the peptide bonds in the protein sample bound to cupric ions in an alkaline medium to form colored peptide/copper complexes.

The SYNCHRON CX System automatically proportioned the appropriate serum sample and reagent into the cuvette. A filled 0.5 ml sample cup was the optimum volume. The ratio used was one part sample to 50 parts reagent. The system monitors the change in absorbance at 410 nm for measurement of alkaline phosphatase activity and at 560 nm for measurement of total protein. These changes in absorbance were directly proportional to the activity of alkaline phosphatase or total protein in the sample and were used by the SYNCHRON CX System to calculate and express alkaline phosphatase activity or total protein concentration.

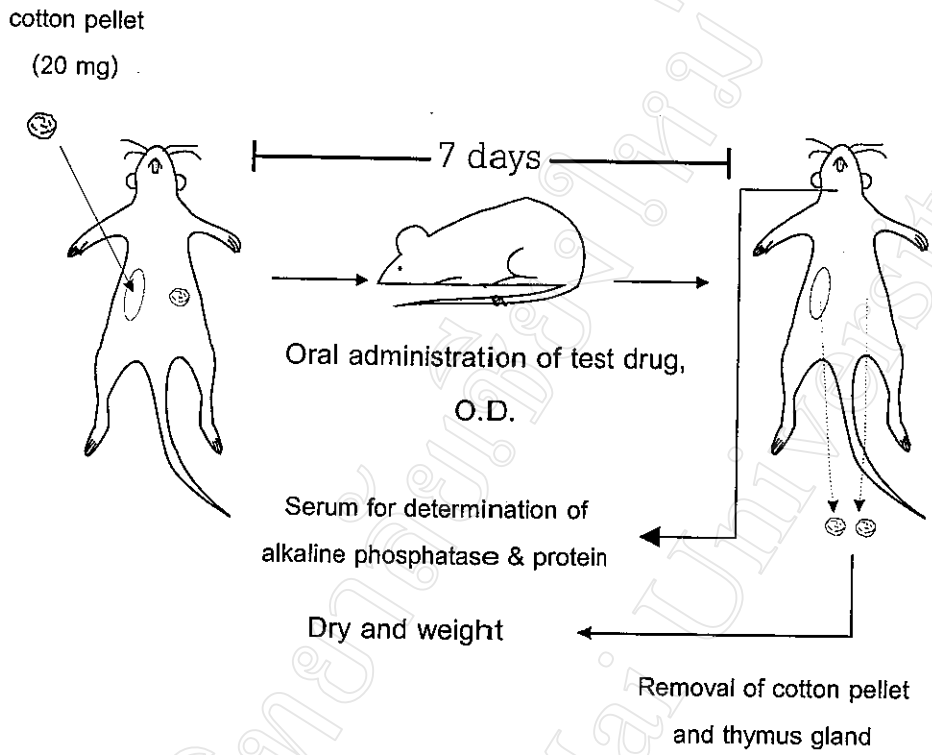


Figure 6. Diagram illustrating the method for cotton pellet-induced granuloma formation in rats

2. Analgesic study

The analgesic activity of PNQ-4482 was tested and compared with standard drugs using two following methods.

2.1 Acetic acid-induced writhing response in mice (Collier, *et al.*,1968 and Nakamura *et al.*,1986).

Male swiss albino mice weighing 30-40 g were used. A writhing response was produced by an injection of an aqueous solution of 0.75% acetic acid in a volume of 0.1 ml/10 g body weight into the peritoneal cavity and the animals were then placed into a transparent plastic box. The number of writhes, a response consisting of contraction of an abdominal wall, pelvic rotation followed by hind limb extension, was counted during continuous observation for 15 min beginning from 5 min after the acetic acid injection.

PNQ-4482, aspirin and morphine were administered intraperitoneally 30 min before the acetic acid injection. The control group received 5% Tween 80. Percent inhibition of writhing response was calculated.

2.2 Formalin test (Hunskaar and Hole, 1987)

The formalin test comprises the early phase and the late phase assessment of the analgesic effect. Male Swiss-Albino mice weighing 30-40 g were injected intraperitoneally with PNQ-4482, aspirin or morphine. The control group received 5% Tween 80.

In the early phase assessment, 20 µl of 1% formalin in NSS was injected subcutaneously into the right dorsal hindpaw of the mouse 30 min after test drugs. Then between 0-10 min after formalin injection, the time in seconds the mice spent for intensive licking the right dorsal hind paw was determined (Figure 7), and percent inhibition of licking response was calculated.

In the late phase assessment, another group of mice was used. The formalin was injected 10 min after test drug treatment and the licking time was determined between 20-30 min after formalin injection (Figure 8).

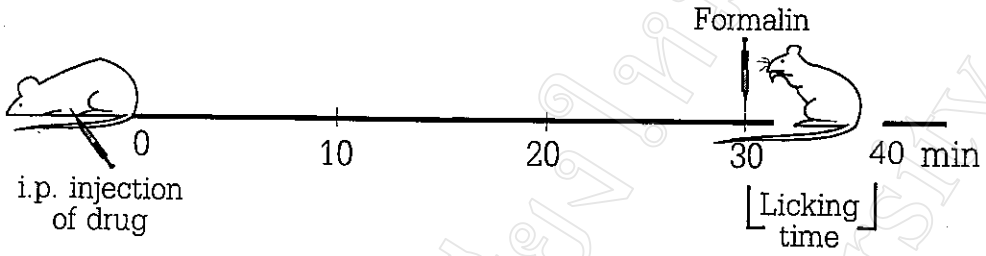


Figure 7. Diagram illustrating the method for formalin test (early phase) in mice

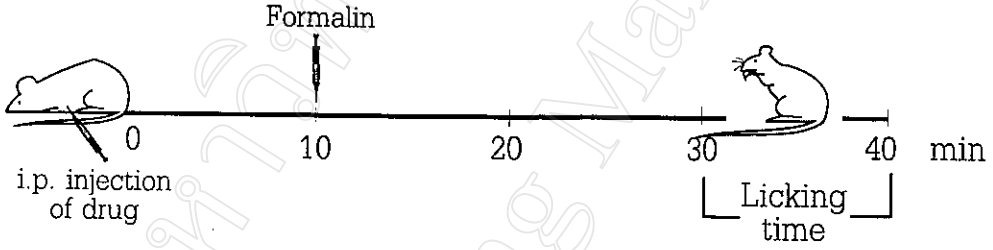


Figure 8. Diagram illustrating the method for formalin test (late phase) in mice

3. Antipyretic study (Teotino *et al.*, 1963)

The antipyretic activity of PNQ-4482 was tested and compared with aspirin. Male rats weighing 280-300 g were used. They were housed and maintained under uniform environmental conditions. Disturbances likely to excite them were avoided. Before pyrexia was induced, the animals were restrained in plastic cages and the initial rectal temperatures were recorded using a ten channel electric thermometer (EXACON, model MC 8940, EXACON Scientific Inturments Aps, Denmark) connected with the probes (model H-RRA, EXACON Instruments Aps, Denmark) which were inserted into the rat rectums to about 5 cm depth. In order to adapt the rats to the handling procedure for probe insertion, the basal rectal temperatures were taken 1 h after probe insertion (Figure 9). Thereafter hyperthermia was induced in rats by subcutaneous injection of 1 ml/100 g body weight of 20% yeast in NSS. When the temperature was at a peak, 18 h after yeast injection, the rectal temperatures were again recorded. Those animals which showed a rise in rectal temperature of more than 1 ^{o}C were used. PNQ-4482 and aspirin were then administered orally and the rectal temperatures of animals were recorded at 30 min interval for 2 h following drug treatment.

4. Anti-ulcerogenic activity study

Preparation of rats for anti-ulcerogenic activity study

Sprague-Dawley rats weighing 180-200 g were fasted 48 h, but had free access of water. The water was withdrawn 1 h before starting the experiment. PNQ-4482, cimetidine or 5% Tween 80 were given orally to the rats 1 h before induction of gastric lesions. The rats were divided into groups of 6 animals.

Group 1	control group; received 5% Tween 80
Group 2	reference group; received a reference drug (cimetidine 100 mg/kg)
Group 3	test groups; received PNQ-4482

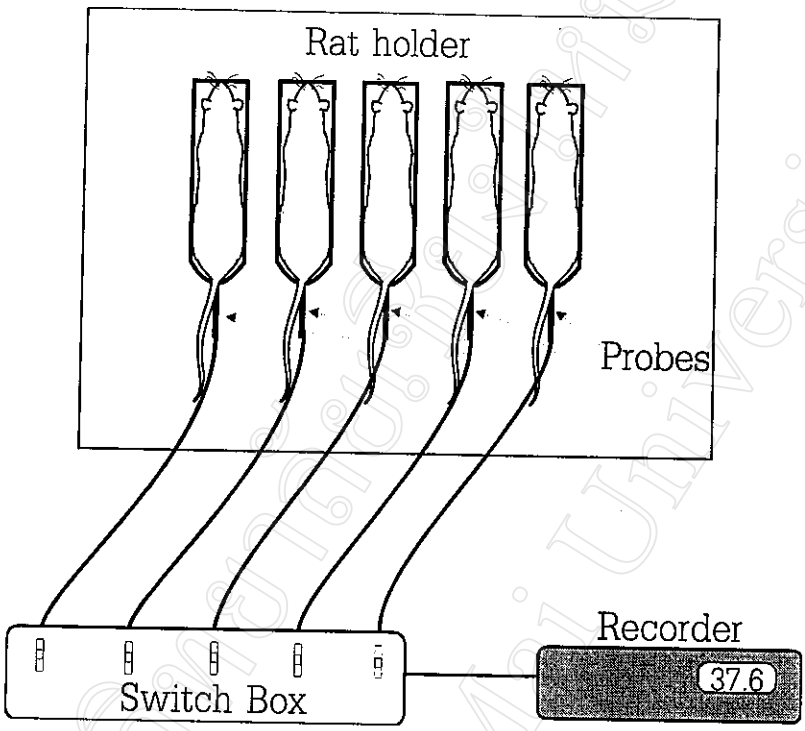


Figure 9. Diagram illustrating the method for yeast-induced hyperthermia

Methods used to induce gastric lesions

4.1 Ethanol/hydrochloric acid-induced gastric lesions (Mizui and Doteuchi, 1988)

Each rat was administered 1 ml of ethanol/hydrochloric acid orally (ethanol 60 ml + hydrochloric acid 1.7 ml + water 38.3 ml). Cimetidine, 100 mg/kg per oral, was used as reference drug. One hour later, the rats were sacrificed and the stomachs were dissected out for determination of gastric ulcers. The scheme of experiment is shown in Figure 10.

4.2 Indomethacin-induced gastric lesions (Pal and Nagohandhury, 1991)

Indomethacin suspended in 5% Tween 80 was injected intraperitoneally at a single dose of 30 mg/kg. Cimetidine, 100 mg/kg per oral, was used as reference drug. Five hours later, the rats were sacrificed for determination of gastric ulcers. The scheme of experiment is shown in Figure 11.

4.3 Restraint water immersion stress-induced gastric lesions (Takagi, *et al.*, 1963)

Rats were restrained in stainless steel cages and immersed up to their xiphoid in a water bath maintained at 20 $\pm$ 2 $^{\circ}$C. Five hours after exposure to water, the rats were sacrificed and the stomachs were dissected out for determination of gastric ulcer.

Evaluation of gastric lesions

After the rats were sacrificed, the stomachs were removed and opened along the greater curvature, rinsed with isotonic saline and pinned out on a wax plate. The glandular portion of the stomach was then examined for lesions.

Gastric lesions: The length (mm) of each lesion was measured under a dissecting microscope (10x). The sum of the total length of lesions in each group divided by the number of rats in that group was expressed as the gastric lesion.

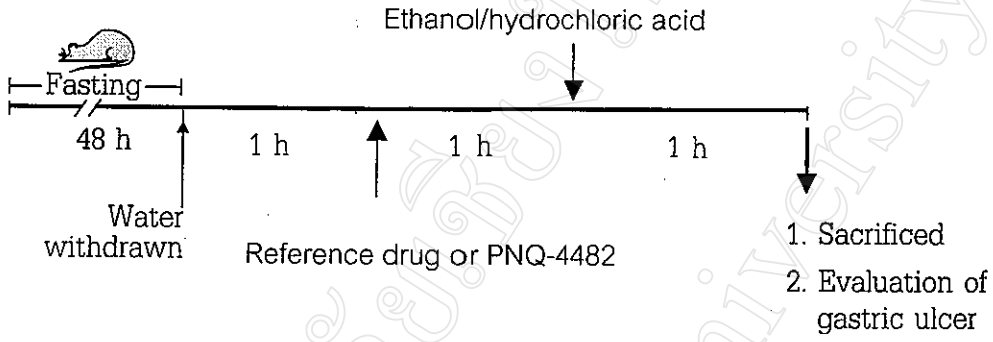


Figure 10. Diagram illustrating the procedure of ethanol/hydrochloric acid induced induced gastric lesion in rats

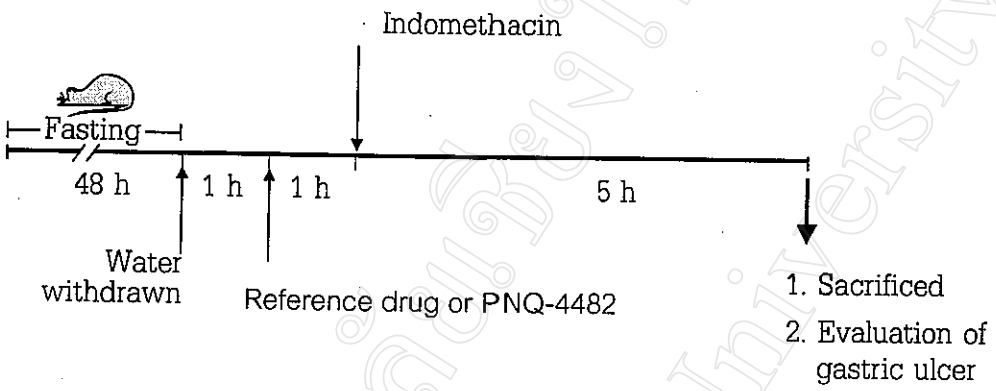


Figure 11. Diagram illustrating the procedure of indomethacin induced gastric lesion in rats

The percent inhibition of gastric ulcer formation was calculated as follows:

$$\% \text{ inhibition} = \frac{G_c - G_t}{G_c} \times 100$$

Where

G = gastric lesions

G_c = gastric lesions of control group

G_t = gastric lesions of test group

4.2.5 Pylorus ligation (Shay, *et al.*, 1945)

Rats were slightly anesthetized by ether. The abdomen was opened and the pylorus ligated with linen thread. Suturing closed the abdomen. Five hours after ligation, the rats were sacrificed. The stomach was removed and gastric content was collected in a graduated centrifuge tube. After centrifugation at 2,500 r.p.m. for 5 min, the volume of gastric juice was measured and the total acidity of the supernatant was determined by titration with 0.1 N NaOH to end point of pH 7.4 using phenolphthalein as an indicator. The scheme of experiment is shown in Figure 12.

Total acidity of gastric juice was calculated as follows:

$$N_1V_1 = N_2V_2$$

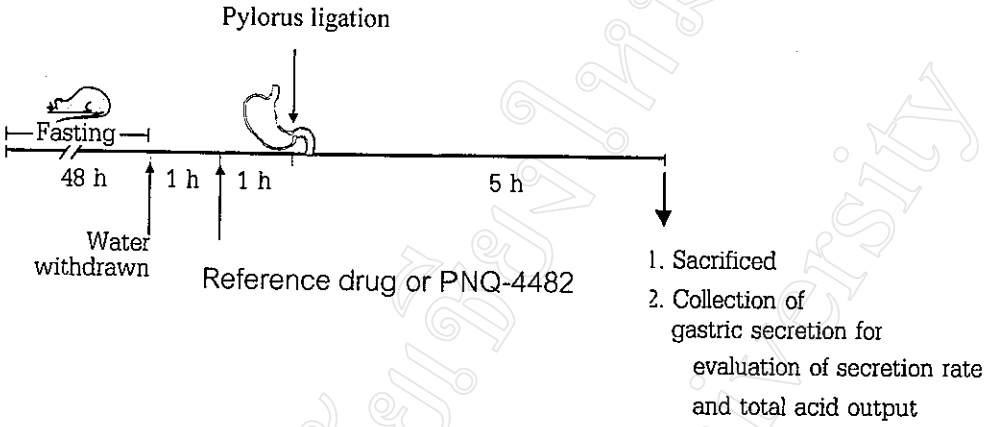


Figure 12. Diagram illustrating the procedure of pylorus ligation in rats

Where

N_1 = normality of gastric juice (Eq)

N_2 = normality of NaOH (Eq)

V_1 = volume of gastric juice (ml)

V_2 = volume of NaOH (ml)

5. Hippocratic screening study

The effect of PNQ-4482 on the general behavior of conscious animals is the objective of this test. The experiment was carried out according to the procedure described by Malone and Robichaud (1962) as follows:

Five dose levels of PNQ-4482 suspended in 5% Tween 80 were given to groups of non fasted rats. A control group received an equal volume of the vehicle intraperitoneally. Four males and four females were used for each dose level. Signs and symptoms observed after the administration of test sample were recorded using a standard working sheet (Figure 13) at 5, 15, 30 minutes, 1, 2 and 24 hours and then once daily for 7 days. Rats which died during the experimental period of 7 days period were autopsied and gross pathological changes of the internal organs (heart, lungs, livers, spleen, kidneys, adrenal glands, sex organs, thymus, brain, eyes, stomach, intestine, etc) were recorded. The surviving animals were sacrificed on the 8^{th} day to examine any gross pathological changes of the internal organs. Any changes of vital organs compared with those of the control group were recorded.

The five dose levels of PNQ-4482 consisted of one lethal dose, three effective doses and one ineffective dose. The lethal and ineffective doses were first determined and then the three effective doses between the two doses were calculated using the following equation.

Date	Qualitative and Semi -quantitative Screening and Toxicity Report of		
Vehicle for sample :	Test Animal :		Fasted ?
Conc :	Sex :	Mark :	Color mark
Ml. inj	Weight (G)		
Route inj	Time inj	Evaluated by :	

Time : (min) post dosage	C	5	15	30	60	120	Time : (min) post dosage	C	5	15	30	60	120
PARAMETER	Response						PARAMETER	Response					
CNS							EARS, ORAL MUCOSA						
Motor Activity							Blanching						
Ataxia							Hyperemia						
Loss.Righting Reflex							Cyanosis						
Analgesia							GENERAL						
Resp. Rate							Salivation						
Resp. Depth							Tail Erection						
Loss. Corneal Reflex							Pilomotor Erection						
Paralysis : legs							Micturation						
Screen grip : H.L. loss							Diarrhoea						
Screen grip : F.L. loss							Robichaud Test						
Fine Body Tremors							Circling Motions						
Coarse Body Tremors							Tail Lashing						
Fasciculations							Abdominal Griping						
Clonic Convulsions							Rectal Temp. C						
Tonic Convulsions							Startle Reaction						
Mixed Type Convulsions													
EYES							Head Tap : Aggressive						
Enopthalmos							Head Tap : Passive						
Exophthalmos							Head Tap : Fearful						
Palpebral Ptosis							Body Touch : Aggressive						
Pupil size, mm.							Body Touch : Passive						
Nystagmus							Body Touch : Fearful						
Lacrimation							Statue Positions						
Bloody Tears							Excess Curiosity						

DEATH AND AUTOPSY NOTES

Figure 13. The standard work sheet for Hippocratic screen

$$F = r\sqrt{I}$$

Where,

F = increment factor

I = interval : lethal dose/ ineffective dose

r = (number of doses in the log series) – 1

6. Acute toxicity study

Rats of both sexes with 150-200 g body weight were used. According to the OECD guideline for testing of chemicals (1981), the test substance was given in a single oral dose by gavage (within the period of 6 h) to rats in treated groups (male 10 and 10 female). One group served as control and received an equal volume of 5% Tween 80. Animals were deprived of food but not water 16 – 18 h prior to administration of the test sample.

Observations were made and recorded systematically 1, 2, 4 and 6 h after test substance administration. The visual observations included changes in the skin and fur, eye and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system as well as somatomotor activity and behavioral pattern. The number of survivors was noted after 24 h and these animals then maintained for a further 14 days with a once daily observation.

At the conclusion of the experiment, all surviving animals were sacrificed and the internal organs such as heart, lungs, livers, kidneys, spleen, adrenals, sex organs and brain were examined.

The toxicity was assessed on the basis of mortality. If a test at one dose level of at least 5,000 mg/kg body weight produces no compound – related mortality, then a full study using three dose levels may not be necessary.

7. Subacute toxicity study

Male and female rats, weighing 140-180 g were used. According to the OECD guidelines for testing of chemicals (1981), three dosage levels of test substance should be given for a period of 28 days. If a test substance at a dose of 5,000 mg/kg orally does not cause death of the animals in the acute toxicity test, a one dose level of 1,000 mg/kg for a period of 14 days can be performed in subacute toxicity and a full study using three dosage levels for 28 days is not necessary. One group of animals served as control and received an equal volume of 5% Tween 80.

During the period of administration, the animals were weighed and observed daily to detect signs of toxicity. Daily visual observations were made and recorded systemically. The observations included changes in the skin and fur, eye and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system as well as somatomotor activity and behavioral pattern. Any rat which died during the test period was examined pathologically.

All surviving animals were fasted overnight but with free access of water and afterwards were anesthetized for collection of blood from a common carotid artery. Blood samples were collected into heparinized and dry non-heparinized centrifuge tubes. Blood analysis (both hematology and chemistry) was carried out. The heparinized blood was used for hematological study included WBC and differential leukocyte count, platelet, hematocrit and hemoglobin estimation. The non-heparinized blood was allowed to coagulate, then centrifuged and the serum was separated. Serum was assayed for glucose, creatinine, BUN, GOT, GPT, alkaline phosphatase, total protein and albumin.

Immediately after blood collection, the animals were sacrificed for tissue studies. The organs such as heart, lungs, livers, kidneys, spleen, adrenals, and sex organs were removed, blotted free of blood and weighed immediately on an electronic balance for subsequent analysis. Eyes, brain, thymus, intestine, uterus, epididymis, seminal vesicles, prostate glands, thoracic spines and muscle with sciatic nerve were also

observed. Histological examinations were performed on the preserved tissues with particular emphasis on those which showed gross pathological changes.

STATISTICAL ANALYSIS

Results were expressed as mean ± standard error of mean (S.E.M.). Statistical significance was determined by one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test. The data obtained from acute toxicity studies were analyzed using Student's *t*-test. P values less than 0.05 were considered significant.

DRUGS AND CHEMICALS

1. Drugs

1.1 Aspirin (acetylsalicylic acid) (Vidhyasom Co., Ltd., Bangkok, Thailand)

1.2 Cimetidine (Tagamet®, Smith & Kline Beecham (Thailand), Bangkok, Thailand)

1.3 Indomethacin (Sigma Chemical Company, St. Louis, U.S.A.)

1.4 Pentobarbital sodium injection U.S.P. (Nembutal®, Abbott Laboratories, North Chicago, U.S.A.)

1.5 Prednisolone (Scherisone®, Schering <Bangkok> Ltd., Nonthaburi, Thailand)

1.6 Phenidone (Riedel-de Haen AG, D-3016 Seelze 1, Germany)

1.7 Phenylbutazone (Sigma Chemical Company, St. Louis, U.S.A.)

1.8 Atropine sulphate (The Government Pharmaceutical Organization, Bangkok, Thailand)

1.9 Chlorpheniramine maleate injection B.P. (The Government Pharmaceutical Organization, Bangkok, Thailand)

1.10 Morphine sulphate injection U.S.P. (The Government Pharmaceutical Organization, Bangkok, Thailand)

2. Chemicals

2.1 Lambda carrageenin (Sigma Chemical Company, St. Louis, U.S.A.)

2.2 Diethyl ether (Merck, Darmstadt, F.R. Germany)

2.3 Glacial acetic acid (J.T. Baker, Phillipsburg, U.S.A.)

2.4 Phenolphthalein (Merck, Darmstadt, F.R. Germany)

2.5 Polyoxyethylene-sorbitan monooleate (Tween 80, Sigma Chemical Company, St. Louis, U.S.A.)

2.6 Sodium hydroxide (Merck, Darmstadt, F.R. Germany)

2.7 Arachidonic acid (Sigma Chemical Company, St. Louis, U.S.A.)

2.8 Ethyl phenylpropiolate (EPP) (Fluka Chemicals Co., Ltd., Japan)

2.9 Acetone (Merck, Darmstadt, Germany)

2.10 Absolute ethanol (Merck, Darmstadt, Germany)

2.11 Hydrochloric acid (BDH Laboratory Supplies Poole, England)

1.11 Methysergide hydrogenmaleate (Sandoz Ltd., Switzerland)