MATERIALS AND METHODS

A. Experiments in vitro

1. Isolated guinea-pig tracheal chain preparation

The isolated guinea-pig tracheal chain was prepared by using the method described in "Pharmacological Experiments on Isolated Preparation" (The staff of the Department of Pharmacology, University of Edinburgh, 1970) as follows:

Guinea-pigs of either sex, weighing 300 to 600 g were used. The animal was killed by a blow on the head and the throat was cut as near the head as possible. The trachea was then rapidly excised and placed in a dish containing oxygenated Krebs' solution (NaCl 5.54, KCl 0.35, $MgSO_4 \cdot 7H_2O$ 0.29, $CaCl_2$ 0.28, KH_2PO_4 0.16, $NaHCO_3$ 2.1 and Glucose 2.1 g/l). After cleaning away the surrounding tissues, the trachea was cut transversely between the segments of the cartilage, to obtain a number of rings. Five rings were then tied together to form a chain with threads. The constant changes in length were assured by offsetting the paries muscle of adjacent rings by 180° as shown in Fig. 3 A. The chain was mounted in a 10 ml tissue bath containing Krebs' solution maintained at 37° C and aerated with oxygen. Mechanical responses were recorded isometrically via a forced-displacement transducer (Grass FT 03b, Grass Instrument Co. Quincy, Mass., U.S.A.) and displayed on a polygraph (79D, Polygraph. Grass Instrument Co. Quincy, Mass., U.S.A.) as shown in Fig. 3 B. The tracheal chains were maintained under the resting tension of 0.75 g and allowed to equilibrate in the organ bath for a period of at

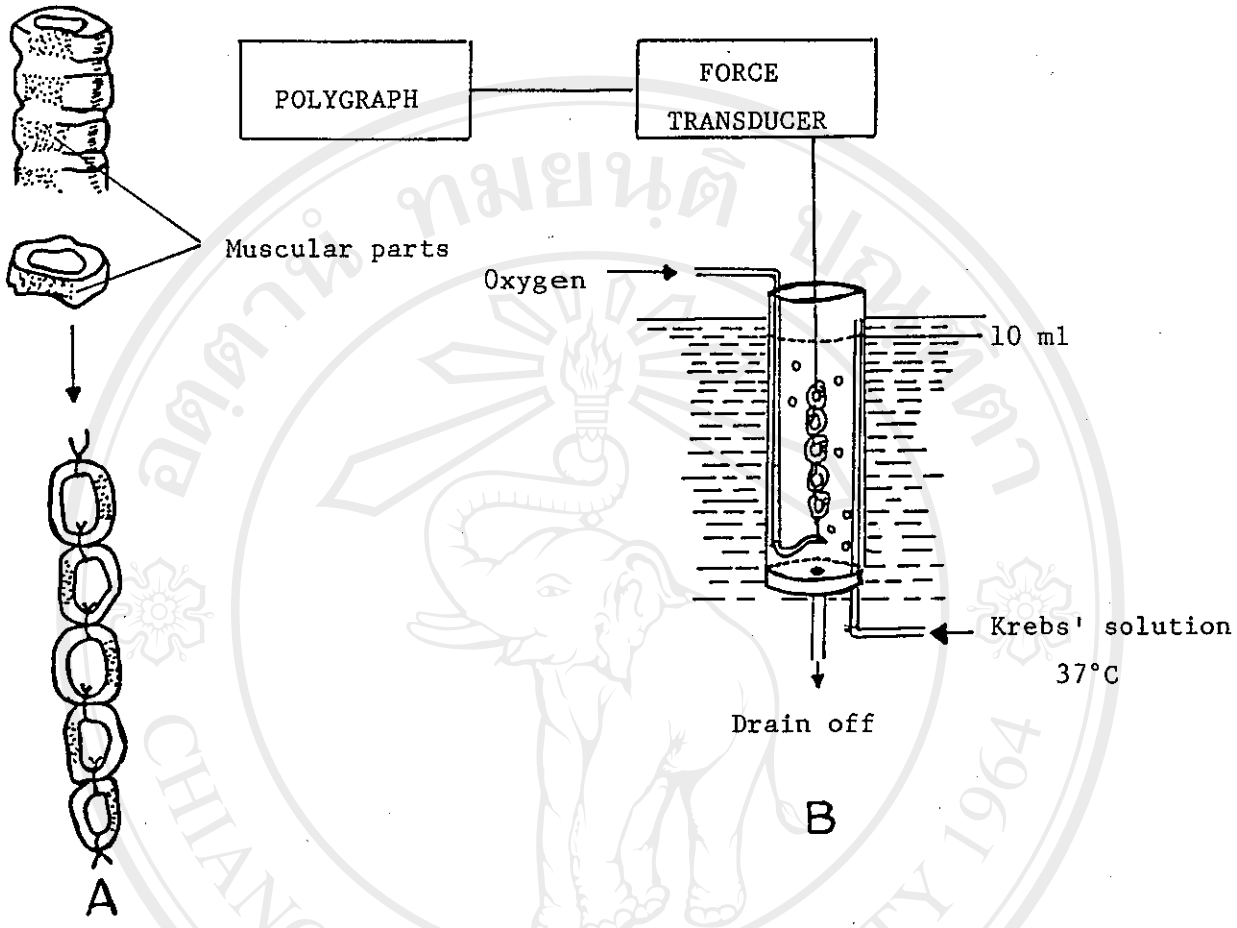

Fig. 3 Experimental set for recording of tracheal contraction using isolated guinea-pig tracheal chain.

least 2 hours. During the equilibration period, the physiological solution was replaced every 30 minutes. The concentrations of the tested drugs used in this study were calculated on the basis of final concentration in the tissue bath. After each dose of tested drug, the tissue was washed 3 - 4 times with fresh Krebs' solution and was allowed to return passively to its resting tension before being reused.

2. Quantitative measurement of tracheal activity

The tracheal contraction induced by a maximal effective concentration of histamine (0.3 ug/ml) (The staff of the Department of Pharmacology, University of Edinburgh, 1970) represents bronchoconstriction. Each successive concentration of phenylalkane derivatives and reference drugs (isoproterenol, aminophylline, atropine, verapamil, papaverine and compound D) was added into the tissue chamber only after the response to an agonist reached a plateau. The antagonistic effect of phenylalkane derivatives and reference drugs was expressed in term of percent relaxation.

$$\% \text{ relaxation} = \frac{\text{response to histamine} - \text{response after adding of tested drugs}}{\text{response to histamine}} \times 100$$

The potency of phenylalkane derivatives was assessed by comparison of their median effective concentration (EC_{50}) values with those of reference drugs. The value of EC_{50} was defined as the concentration of tested

drugs which induced a 50 % relaxation of the agonist-induced tracheal contraction.

3. Determination of the mechanism of action of phenylalkane derivatives

The possible mechanisms of action of phenylalkane derivatives were investigated by:

3.1 Comparison of the dose-response relationship of phenylalkane derivatives with those of reference drugs on histamine-induced tracheal contraction.

By employing a least Square method, the dose-response relationship between various doses of phenylalkane derivatives or reference drugs and their percent relaxation of the histamine-induced tracheal contraction was expressed as a linear regression equation, $Y = a + bX$. The slopes were tested for parallelism.

3.2 Comparison of the antagonistic effect of phenylalkane derivatives and reference drugs on the histamine-induced tracheal contraction in the presence of propranolol (β-adrenergic antagonist).

Doses of reference drugs and phenylalkane derivatives which caused maximum relaxation of a histamine-induced contraction were used. The dose of propranolol which could effectively block the effect of isoproterenol was determined and used to study its blocking effect on the reduction of histamine-induced contraction caused by aminophylline, verapamil, papaverine and phenylalkane derivatives. Propranolol was added into the tissue bath 3 min before challenge with histamine. The antagonistic

effect of the tested drugs was then evaluated when the tracheal contraction reached a plateau. The effect of tested drugs on established contraction was recorded both when propranolol was absent and present.

4. The rat tracheal strip preparation

The methods employed were essentially similar to those previously described by Wellens (1966) and Lulich and Paterson (1983) with slight modification as follows:

Albino rats of both sexes, weighing 200 to 250 g were used. The animal was killed by a blow on the head. The trachea was immediately removed into a dish containing oxygenated Krebs' solution. Surrounding tissues were carefully removed from each trachea without damaging the smooth muscle. The trachea was then divided into two portions, proximal and distal, each being 0.5 cm long. Each portion was cut longitudinally through the cartilagenous region opposite the muscle part and then tied at each end of the strip with thread (Fig. 4 A). The tracheal strip was placed in a tissue bath containing a 10-ml aerated Krebs' solution and the temperature was maintained at 37°C. The isometric contraction of the tracheal strip was recorded via a force displacement transducer and displayed on a polygraph as shown in Fig. 4 B. A resting tension of 0.75 g was established and the strip was equilibrated for 1 hour before starting the experiment.

In this study, methacholine was used as a bronchoconstrictive inducer. The dose of methacholine which caused the submaximal contraction of the tracheal muscle was first determined and used to induce bronchoconstriction. The effect of phenylalkane derivatives and reference drugs (aminophylline, atropine, verapamil, papaverine and compound D) were tested when the agonist-induced contraction reached a plateau. The bronchodilator effect of tested drugs was expressed in term of percent relaxation, which was calculated as described in 2.

The dose-response relationship and relative potency of phenylalkane derivatives and reference drugs were assessed as decribed in the previous experiment of the guinea-pig tracheal chain.

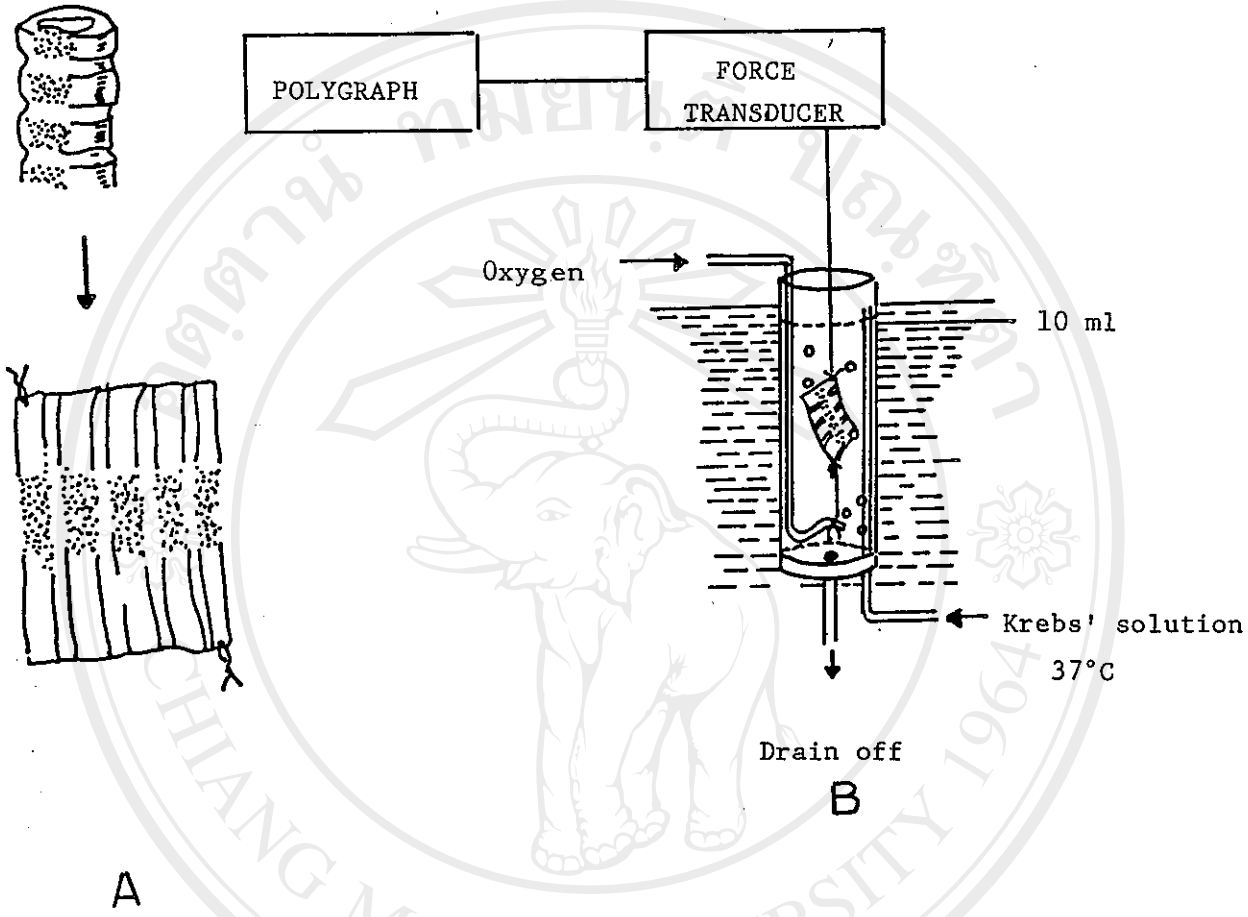

Fig. 4 Experimental set for recording of tracheal contraction using isolated rat tracheal strip.

B. Experiment in vivo

1. Histamine-induced bronchoconstriction in pentobarbital anesthetized guinea-pigs.

The experiment was set up according to the method of Salonen and Mattila (1981, 1985) and slightly modified as follows:

Guinea-pigs of both sexes weighing 300 to 400 g were used. The animal was anesthetized with pentobarbital sodium (40 mg/kg body weight, intraperitoneally). The polyethylene tube was inserted into the trachea and the animal was artificially ventilated with a Harvard rodent ventilator (680 model, Harvard Apparatus Co., Dover, Mass., U.S.A.) at a ventilation rate of 72 strokes/min and a stroke volume of 7 ml/kg body weight. The right jugular vein was cannulated with a polyethylene cannula for injection of tested drugs. To prevent spontaneous breathing, 2 mg/kg body weight of pancuronium bromide was given intraperitoneally. The test animals usually showed no signs of spontaneous breathing about 10 min after the administration of the muscle relaxant. During the experiment, no extra doses of anesthetic or relaxant were given. Animals were discarded if they failed to show full anesthesia with complete muscle relaxation. Intratracheal pressure was measured by connecting a side arm of the tracheal cannula to a bronchospasm transducer (720 model, Ugo Prasile, Italy) the output of which was recorded on a polygraph as shown in Fig. 5. The amplitude of the penwriter was adjusted to give 2ml = 1cm. An increase in intratracheal pressure was taken to denote bronchoconstriction.

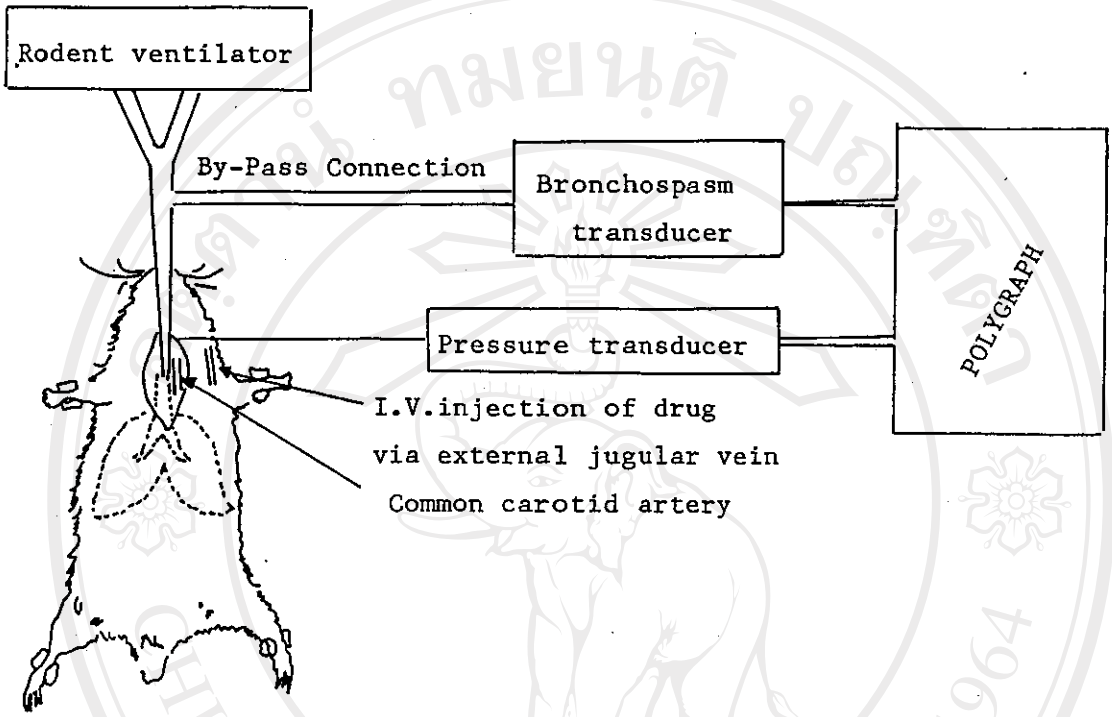

Fig. 5 Experimental model for recording of intratracheal pressure in pentobarbital anesthetized guinea-pig or rat.

In each guinea-pig, two supramaximal doses of histamine (8 ug/kg body weight) were used to induce bronchoconstriction (Kansenalak, 1985 and Srisawadee, 1987). Only animals which responded to the first dose of histamine were used. The second dose of histamine was injected 20 min after the first dose. Phenylalkane derivatives and reference drugs were given 2 min before the second dose of histamine. After each dose of the drugs, the polyethylene catheter was rinsed with 0.1 ml of NSS.

The common carotid artery was cannulated with polyethylene cannula, which was filled with heparinized saline (60 units/ml isotonic solution). The arterial blood pressure was concurrently recorded via a pressure transducer (Statham P 23 AC Straingauge transducer, Laboratories Inc., Hato Rey Puerto Rico) and displayed on a polygraph.

2. Quantitative measurement of bronchial activity

In order to measure quantitative bronchoconstriction, the difference in the penwriter amplitude between the peak height of the intratracheal pressure response (PIPR) and the basal resting value was measured in millimeters for each histamine dose. An increase in the response area (RA) was also measured for the two-min period after each dose by using a transparent millimeter paper, and the values were expressed in square millimeters. A single animal was used for a single dose of each tested drug. The bronchodilator activity of tested drugs was expressed in terms of percent inhibition of PIPR and RA to the second dose of histamine challenge and calculated as follows:

$$\% \text{ inhibition of histamine induced bronchoconstriction} = \frac{\text{bronchoconstriction induced by histamine} - \text{bronchoconstriction induced by histamine after pretreated with tested drugs}}{\text{bronchoconstriction induced by histamine}} \times 100$$

3. Methacholine-induced bronchoconstriction in pentobarbital anesthetized rats.

The method described by Salonen and Mattila (1981) was used as follows:

Albino rats of either sex, weighing 220 to 260 g were used. The animal was anesthetized with pentobarbital sodium (50 mg/kg body weight intraperitoneally). A polyethylene cannula was inserted through a tracheostomy and artificially ventilated with a stroke volume of 10 ml/kg body weight and rate of 70 strokes/min,using a Harvard rodent ventilator. Pancuronium bromide (2.5 mg/kg body weight) was given intraperitoneally to prevent spontaneous respiration of the animal. The intratracheal pressure and the blood pressure were recorded in the same way as previously described in the experiment using guinea-pigs (Fig. 5). A set of doses of

methacholine (MeCh) was injected (1.5, 3 and 4.5 ug) at 2 min intervals into the right jugular vein to induce bronchoconstriction. Only animals which responded to the first set of cumulative doses of MeCh were used. Twenty minutes after the first set of MeCh doses the animal was given a second set of MeCh doses, 3, 6 and 9 ug, injected similary at 2 min intervals. Phenylalkane derivatives and reference drugs were given intravenously into the right jugular vein of the rat 2 min before administration of the second set of cumulative MeCh doses. After each dose of the drugs, the polyethylene catheter was rinsed with 0.1 ml of NSS.

The assessment of bronchodilator activity of phenylalkane derivatives and reference drugs was performed similary to those described in the guinea-pig experiment.

4. Hippocratic screening test

Effect of phenyl alkane derivatives on the general behaviours of conscious rats was studied. The experiment was carried out according to the procedure described by Malone and Robichaud (1962) as follows:

Non-fasted rats weighing 160 - 180 g were used. Phenylalkane derivatives were suspended in 0.5% methyl cellulose, except PA-3 which was dissolved in normal saline, and given to animals intraperitoneally.

Signs and symptoms induced by test substances were observed at 5, 15 and 30 min and at 1, 2, 4 and 6 hours and recorded on the standard work sheet (Fig. 6). At least four animals were used for complete evaluation at each dosage level.

After 7 days observation, all surviving animals were killed and autopsied in order to observe the internal organs (i.e. liver, kidney, heart) and compared with those of control animals. The aqueous vehicle used in the control animals was 0.5% methyl cellulose and NSS.

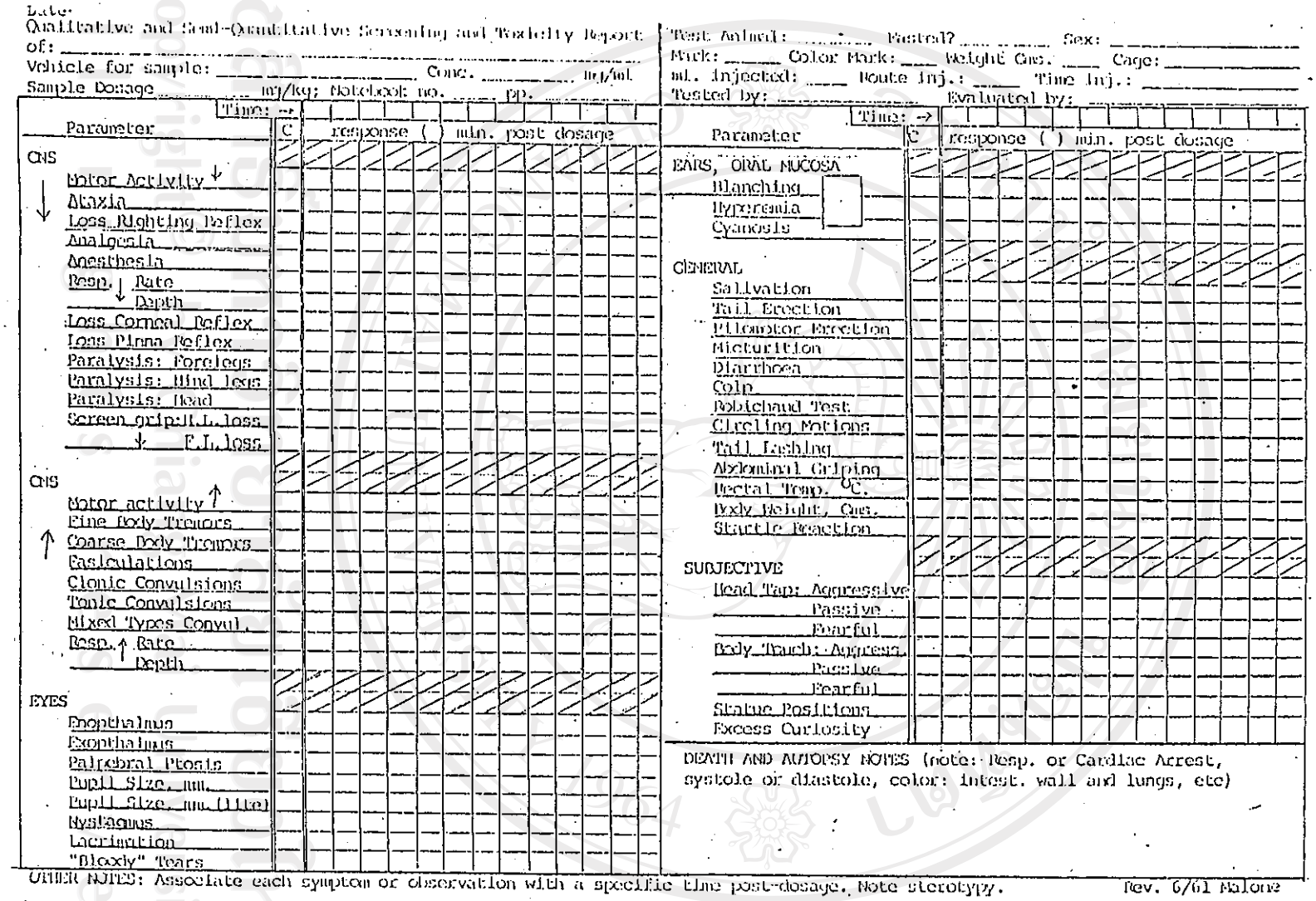

Fig. 6 Standardized work sheet for Hippocratic screening test

C. List of drugs and solvents

1. Ethanol absolute GR (J.R. Bager Chemical B.V., Holland)
2. Aminophylline, 250 mg/ 10ml ampule (Atlantic Laboratories, Thailand)
3. Atropine sulfate U.S.P. XIX, 0.60 mg/ml ampule (the Government Pharmaceutical Organization, Thailand)
4. Heparin 5000 i.u./ml (Leo Pharmaceutical product, Ballerup, Denmark)
5. Histamine dihydrochloride, B grade (Calbiochem, Los Angeles, U.S.A.)
6. Isoproterenol HCL U. S. P., 0.2 mg/ml ampule (Isuprel^{R} HCL, Winthrop - Breon laboratories, U.S.A.)
7. Methacholine chloride (Acetyl-B-Methylcholine chloride) (Sigma^{R} Chemical Company; ST. Louis, U.S.A.)
8. Methyl cellulose (Mathocel MC^{R} , Fluka AG, CH-9470 Buchs, Nur für Laborzwecke gepruft)
9. Pancuronium bromide, 2 mg/ml ampule (Pavulon^{R} , Organon Oss, Holland)
10. Papaverine hydrochloride (Ingeheim am Rhein, Germany)
11. Pentobarbital sodium, 50 mg/ml (Nembutal^{R} sodium, Abott Laboratories, U.S.A.)
12. Propranolol HCL, 1 mg/ml ampule (Inderal^{R} , I.C.I. Macclefield, Great Britain)
13. Verapamil hydrochloride, 5 mg/2 ml ampule (Isoptin^{R} , Knoll AG, Germany)

D. Statistical analysis

The data obtained was analyzed by using a computer programme (SPSS-X RELEASE 3.01 COMPUTER SERVICE CENTER C.M.U.) and the following methods were used in this study:

1. Correlation coefficient value (r), for measuring the association of dosage and response.
2. Linear regression equation ($Y = a + bX$), for expressing the relationship between doses and responses.
3. Median effective concentration (EC_{50}) or median effective dose (ED_{50}), the concentration or dose which produced 50% response.
4. Student's t-test, for assessing the significance of differences between means.
5. ANOVA, for analyzing parallelism between dose-response curve of tested drugs.