CHAPTER 3

MATERIALS AND METHODS

1. Experimental animals

All experiments were carried out in adult guinea-pigs. Guinea-pigs of either sex, weighing 400-600 g were supplied from the animal house, Faculty of Science, Prince of Songkla University. They were housed in an air-conditioned room (24-26 ◦ C) with a 12 hr light/dark cycle (light on 8 AM; light off 8 PM) and the relative air humidity (50±5%) was strictly regulated. Food and water were available *ad libitum* to all animals.

2. Drug pretreatment

Guinea-pigs were divided into control and cocaine treated groups as follows.

2.1 Control groups

The animals in this group received 1 ml/kg of 0.9% w/v sodium chloride solution via intraperitoneal (i.p.) twice a day for 14 days. After the last injection of the saline solution, the animals were sacrificed by cervical dislocation at 24 hour.

2.2 Cocaine treated groups

Guinea-pigs were chronically treated with cocaine hydrochloride as described by Darmani et al., (1992). They were injected with cocaine hydrochloride 2.5 mg/kg intraperitoneally twice a day for 14 days, in the same way as control groups. After cocaine cessation, the animals were also killed at 24 hours.

3. Experimental protocol

Part A studies on guinea-pig isolated atria and trachea

After the cessation of saline or cocaine treatment as mentioned above, the guinea-pigs were killed by cervical dislocation and exanguinated at 24 hours. The atria and trachea were carefully isolated for studying of their contraction and their relaxations, respectively, in responses to exogenous applied catecholamine, epinephrine and non catecholamine, salbutmol.

1. Isolated atrial preparations

After guinea-pigs were killed, their hearts were quickly dissected out and immersed in Krebs-Henseleit (Krebs') solution as described by Grassby and Broadley (1986). After removal of the pericardium, ventricular tissues were separated from atria. The isolated atria were then suspended in a 25-ml organ bath containing 37 ◦ C Krebs' solution. The baths were gassed continuously with a mixture of 95% O2 and 5% CO_2 (Figure 9 and 10). The atria were set up under initial resting tension of 2 g. Changes in atrial tension were recorded isometrically with a force displacement transducer (Grass Instrument CO., Quincy, Mass., U.S.A) connected to a Grass model 7 D polygraph. The change in tension signal was used

to trigger a Grass tachograph so that recording of atrial tension and rate of beating were made simultaneously. Before the onset of each experiment, the atrial preparation was equilibrated for at least 45 minutes and the Krebs' solution in the organ bath was changed every 10 minutes in order to stabilize the base-line value. Each experiment was repeated in at least 8 isolated atria.

2. Isolated tracheal preparations

Guinea-pig tracheas from cervical to thoracic portion were dissected out.

The tracheal preparations were very cautiously cleaned of unnecessary adipose and connective tissue and to ensure that the smooth muscle was not damaged. The trachea was opened by cutting longitudinally at the hyaline cartilage region which was opposite to the tracheal smooth muscle (Ito et al., 1995). A segment of trachea, 2-3 cm in length, was zigzag cut at each two or three cartilaginous rings as shown in Figure 11 (Staff, Department of Pharmacology, Prince of Songkla University, 1995). The tracheal preparation was mounted in a 25 - ml organ bath containing Krebs' solution maintained at 37 ◦ C and gassed with 5% CO_2 in O_2 (Figure 12).

Preparations were suspended using stainless steel hooks in a 20-ml organ bath. The tissue was subjected to an imposed tension of 2 g and equilibrated for at least I hour before starting the experiment, during which the Krebs' solution was replaced with fresh Krebs' solution every 10 minutes. After equilibration, the tracheal preparation was induced to contract by being exposed to 1 μg/ml carbachol for 30 minutes. A stable contractile response of the trachea was observed before the beginning of each experiment. Change in tracheal tensions were isometrically recorded with a force displacement transducer (Grass Instrument, Quincy, Mass.,

U.S.A.) connected to an amplifier of Grass model 7D polygraph. Each experiment was repeated at least 8 isolated tracheas.

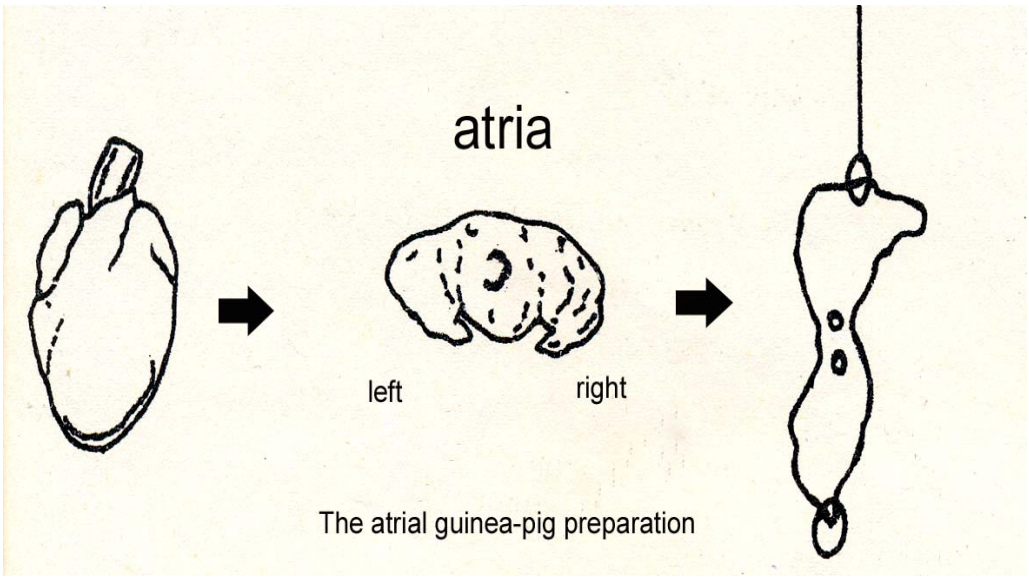


Figure 9 The isolated guinea-pig *atrial* preparation.

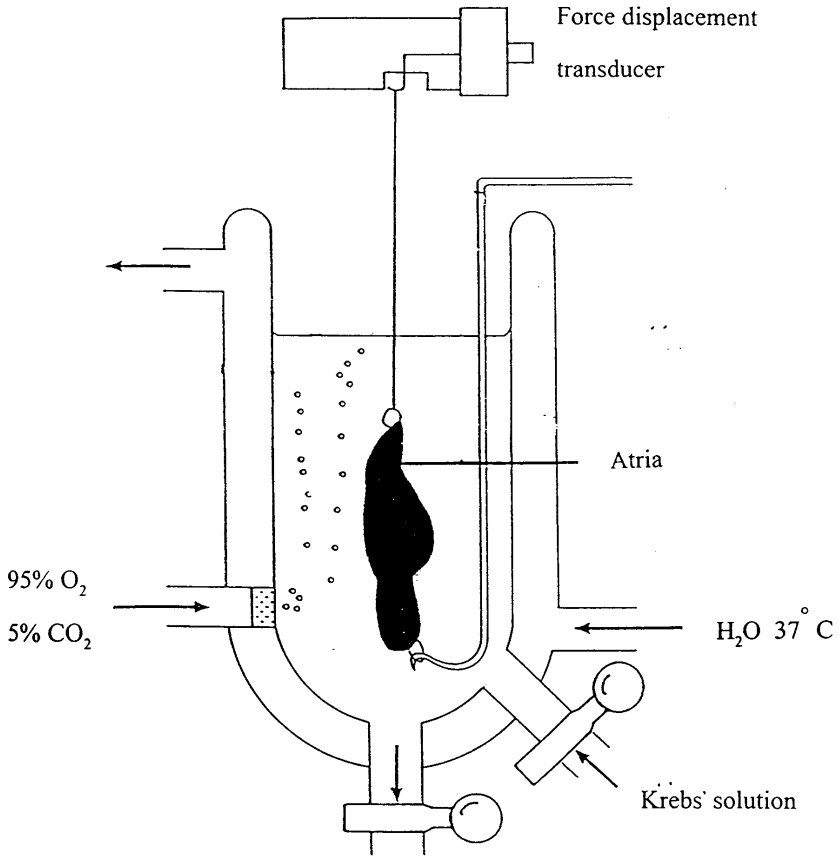
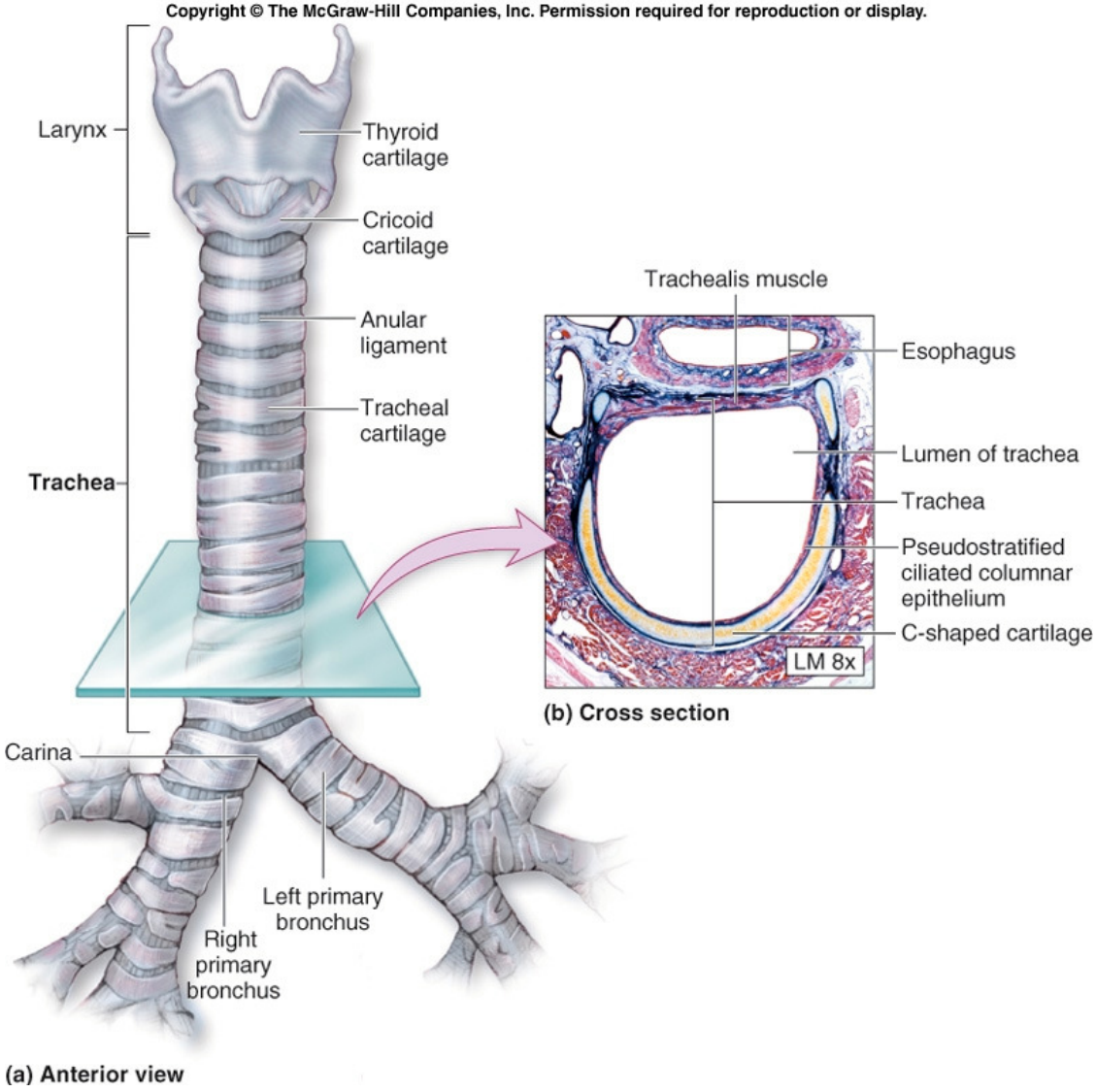


Figure 10 Set up of isolated guinea-pig *atria* for recording of their contraction



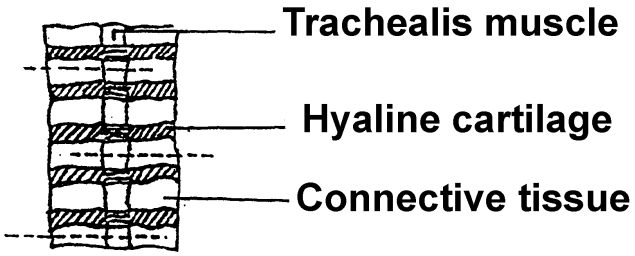


Figure 11 Diagrams show the segments of *trachea* dissected from guinea-pig (above) and preparation of tracheal strip by zigzag cutting (below)

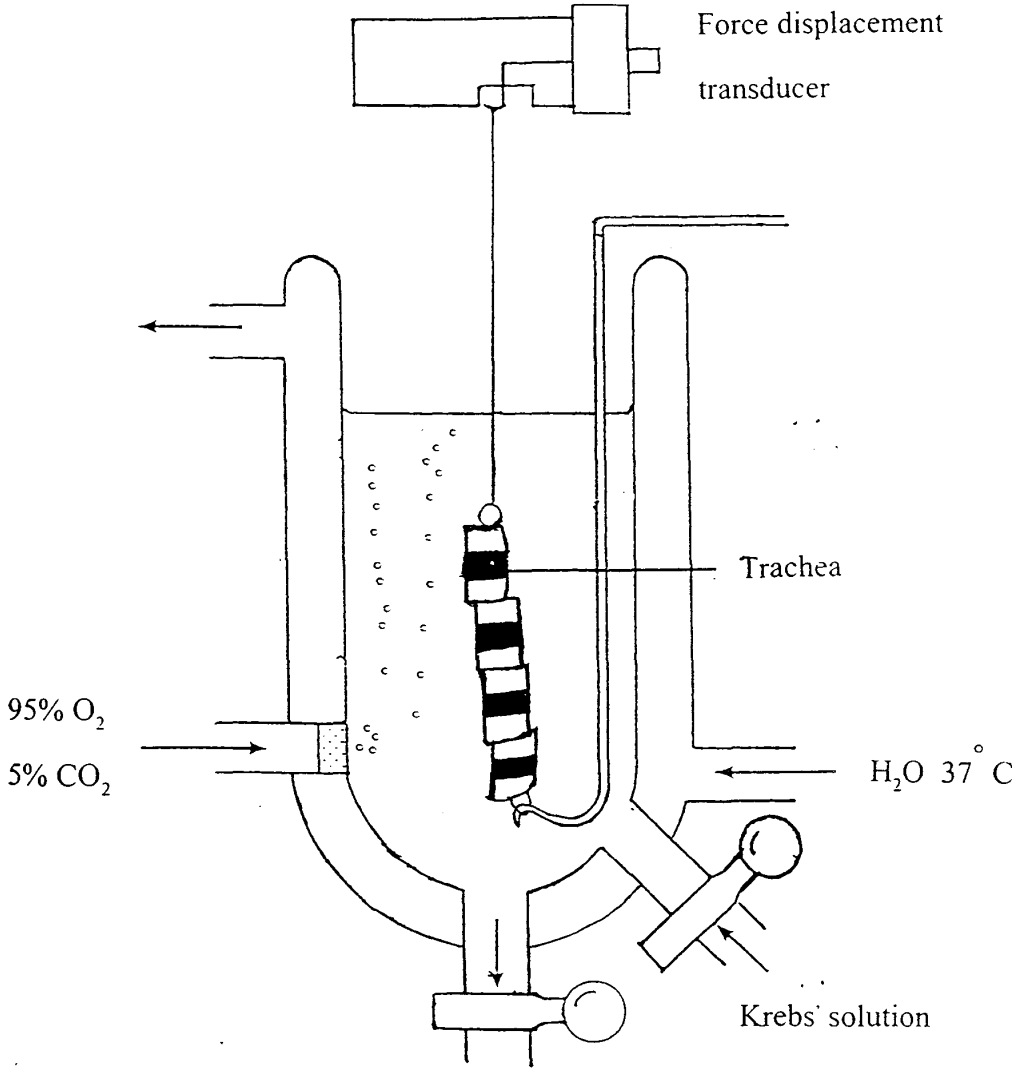


Figure 12 Set up of isolated guinea-pig *trachea* for recording of its contraction or relaxation.

3. Experimental procedure

3.1 Determination of agonistic activity

The isolated atria and tracheas from cocaine-treated and control saline-treated animals were concomitantly studied in order to compare their responsiveness to adrenergic agonists.

3.1.1 Determination of inotropic and chronotropic responses to adrenergic agonists of the guinea-pig isolated atria

After the equilibration period, epinephrine or salbutamol was added to the bathing solution in a cumulative increase in concentration manner. The two drugs were administered in a three-fold increase in concentration. The interval between each addition was adjusted to allow the effect of each concentration to develop fully. The total volume of drug added was kept as small as possible with maximum allowance volume of 1 ml. The inotropic and chronotropic effects of each concentration of the adrenergic agonists were recorded and the concentration-response curves were constructed

3.1.2 Determination of the relaxation responses to adrenergic agonists of the guinea-pig isolated trachea

Prior to the studies of the effects of epinephrine or salbutamol, the trachea was induced to contract by pretreatment with carbachol (1 μg/ml). After the maximum contraction was

obtained, epinephrine or salbutamol was added into the bathing fluid in the cumulative increase in concentration schedule as those described for isolated atria but in a ten-fold increase in concentration. The relaxing effect of each concentration of the two drugs was recorded and the concentration-response curves were prepared.

3.2 Determination of antagonistic activity

The cumulative concentration-response curves of the isolated guinea pig atria or trachea to agonist (epinephrine) were obtained, and the tissues were then incubated with the antagonist, propranolol (10^{-8} M) for 15 min. The cumulative concentration-responses to epinephrine were determined again. The procedure was repeated using five more concentrations ($3x10^{-8}$- $3x10^{-6}$ M) of antagonist. The concentration-response curves of epinephrine in both the absence and presence of propranolol were constructed and compared.

4. Drugs and chemicals

Drugs used in this part of experiments were epinephrine (+)-bitartrate, salbutamol hemisulfate, (±)-propranolol HCL and carbamylcholine chloride (carbachol). They were purchased from Sigma Chemical Company (St. Louis, U.S.A). Cocaine hydrochloride was obtained form Diosynth (Apeldoom, Netherlands). All agents, except cocaine hydrochloride, were prepared as stock solution (0.1 M for epinephrine and salbutamol and 1 mg/ml for carbachol) in 0.1% ascorbic acid in water. Cocaine hydrochloride was dissolved in 0.9% sodium chloride solution. All drugs were kept at -4◦ C until use. For each day of experiment, working solutions were freshly diluted from the stock solutions

with Krebs' solution to appropriate concentration. The Krebs-Henseleit (Krebs') solution had the following composition (mM): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.9; $NaHCO_3$, 25; $MgSO_4$ $7H_2O$, 1.2; KH_2PO_4, 1.2; D-glucose, 11.7 and ascorbic acid, 0.14. All chemicals were analytical grades.

5. Data analysis

Data were expressed as means ± standard error of means ($\overline{X}$ ± S.E.). For each group, the concentration-response curves were plotted. Changes in tension from the resting level were measured at each concentration of agonist and plotted. The plots were also constructed as a percentage of the maximum responses. Regression lines were fitted to the linear portion of the log concentration response curves of epinephrine and salbutamol by the method of least squares. $[D]_{max50}$ values (concentrations required to produce 50 % of their own maximum effects) were calculated. Regression lines of the responses of atria and trachea from cocaine-treated groups and those corresponding control saline-treated groups were tested for parallelism and their $[D]_{max50}$ were compared. The pD_2 values (the negative logarithm of the $[D]_{max50}$) were also determined.

The analyses also were performed on the individual $[D]_{max50}$ obtained from each concentration-response curves for epinephrine and salbutamol of the preparation from both cocaine-treated and saline-treated guinea-pigs using unpaired Student's *t*-test. *P* value less than or equal to 0.05 was considered to indicate statistically significant differences. The changes in the responsiveness to epinephrine and salbutamol of the atria and trachea isolated from the cocaine-treated and the control guinea-pig were also determined using $[D]_{max50}$ ratio ($[D]_{max50}$ of the two drugs of the control-saline treated groups divided by those $[D]_{max50}$ of

cocaine-treated groups). The increase in force of contraction, heart rate, and tracheal relaxation between cocaine- and saline-treated of each drug concentration was determine using analysis of variance (ANOVA) followed by Scheffe or Dunneth's C test to determine individual difference. The difference was considered to be significant when $p < 0.05$

The antagonistic potency of propranolol was expressed as a pA_2 value. The pA_2 values were calculated from the concentrationratios according to the method originally reported by Arunlakshana & Schild (1959). Antagonist was considered to be competitive if the slope of the regression of Schild plot was not significant different from unity. The pA_2 values of propranolol against the effect of epinephrine on atria and trachea of the cocaine-treated and control, saline-treated guinea-pigs were compared using unpaired Student's *t*-test. The *p* value of less than 0.05 was considered to be statistically significant.

Part B Analysis of cocaine concentration in plasma, cardiac, and tracheal tissues

1. Animals and protocol

Guinea-pigs were chronically pretreated with either 0.9% sodium chloride solution or cocaine hydrochloride (2.5 mg/kg) as described above. After cessation of cocaine or sodium chloride

solution, animals were killed at 24 hr. Five milliliters of blood was collected from the heart and put into a heparinized glass tube containing 250 units of heparin sodium. The plasma was separated by centrifugation at 3,500 rpm for 15 minutes. The heart and trachea were also dissected. Atria 200 mg and ventricle 1000 mg were separated and homogenized by grinder tissue homogenizer in 5 ml of 0.9% sodium chloride except tracheal smooth muscle 858 mg (total 10 n) homogenized by grinder tissue homogenizer in 1 ml of 0.9% sodium chloride (Robert et al., 1991). One hundred milligrams of sodium fluoride was added into plasma, homogenated cardiac tissues and tracheal smooth muscle in order to maintain the stability of cocaine (Isenchmid et al., 1989). All samples were stored at -20 ◦ C until analysis.

2. **Chemicals and reagents**

Cocaine hydrochloride was obtained from Diosynth (Apeldoom, Netherlands). Acetonitrile (HPLC grade), sodium fluoride (analytical grade and disodium hydrogen phosphate (analytical grade) were purchased form JT Baker Inc. (Philipsburg. NJ, U.S.A.). Analytical grade acetic acid, sodium dihydrogen phosphate, ammonium acetate, chloroform and 2-propanol were purchased from Merck (Darmstadt, F.R. Germany). Sterile water was obtained by deionization and filtration through a Milli-Q and Nanopure system (Millipore, Molsheim, France), respectively.

3. Instrumentation and chromatographic condition

The HPLC system consisted of a liquid chromatograph, an automatically pump (Waters 515 HPLC pump), an automatically injector (717 plus autosampler) and an ultraviolet detector (Waters 2487). Detection was made with the variable wavelength UV detector set at 235 nm and peak area was measured with the csw32 program of Waters. Chromatographic separation was performed on a Novapak C_{18} column, particle size 4.5 μm, 3.9 x 150 mm I.D. (Waters Associated, Milford, U.S.A.). A guard-pak precolumm model was used to obviate the effect of rapid column degeneration.

4. **Analytical method**

Cocaine concentration in plasma, atrial, ventricular, and tracheal tissues were assayed by a high performance liquid chromatographic (HPLC) method modified from that described by Lim and Peters (1984).

4.1 Mobile phase

The mobile phase consisted of 300 ml of acetonitrlie and 700 ml of water containing 0.1 M ammonium acetate (adjusted to pH 5.15 with glacial acetic acid). The mobile phase was freshly prepared daily, filtered through 0.45 μm filter paper (Peerce, Rock Ford, IL, U.S.A.) and degassed before use. The flow rate was 1.0 ml/min, which gave the pressure of 1,800-2,000 psi. All analyses were performed at room temperature (23-27 ◦C).

4.2 Stock solution

Stock solution of cocaine hydrochloride (1 mg/ml) was prepared by dissolving 10 mg of standard cocaine hydrochloride in methanol and adjusted to the volume of 10 ml in volumetric flask. The stock solution was stored at-20°C. To assay precision and the percentage recovery, working standard solutions of cocaine (50, 100 and 500 ng/ml) were prepared by dilution of the stock standard solution with deionized water, drug-free plasma, atria or ventricular tissue homogenates. The addition of sodium fluoride to all samples to prevent cocaine hydrolysis was necessary.

4.3 Extraction procedure

Two hundred microlitters of plasma, atrial, ventricular tissue homogenates were extracted by adding 150 μl of 0.1 M, Na_2HPO_4 (pH 8.9) and 5 ml of chloroform: 2-propanol (9:1). After vortex mixing for 2 min and centifuging at 3,500 rpm (about 700 g) for 10 min, 4 ml of the organic phase were evaporate to dryness under air stream and the residue was reconstituted with 500 μl of 0.05 M NaH_2PO_4 (pH 5.2) and the content was transferred to a polypropylene microcentifuge tube. After centrifugation for 10 min, 100 μl of the supernatant was injected onto the HPLC system (Tagliaro et al., 1994).

4.4 Calibration procedure

Standard calibration was run on each day of analysis. Calibration samples of cocaine were obtained by adding standard cocaine hydrochloride solution to drug-free plasma, atrial, ventricular and tracheal tissue homogenates and adjusting cocaine concentration to 6.25, 12.50, 25, 50 and 100ng/ml. The spiked plasma, atrial, ventricular and tracheal tissue samples were extracted and assayed as described above. The peak areas obtained from the chromatograms were plotted against cocaine concentration. The calibration curves of cocaine in plasma, atrial, ventricular and tracheal tissues were illustrated in Figure 13-16.

4.5 Limit of Detection (LOD)

The detection limits which is defined as the lowest concentration of cocaine hydrochloride in plasma and homogenated tissues that can be detected, were calculated as follows:

$$LOD = 3.3\sigma/S$$

where σ = the stanndard deviation of the response (S.D.)

S = the slope of calibration curve

4.6 Limit of Quantitation (LOQ)

The limit of quantitation (LOQ) is defined as the lowest concentration of cocaine hydrochloride in plasma and homogenated tissues that can be determined with acceptable precision and accuracy under the stated operational conditions of the method.

$$LOQ = 10\sigma/S$$

where σ = the standard deviation of the response (S.D.)
S = the slope of calibration curve

4.7 Assay precision and variability

The within-day, between-day precision and variability were determined using plasma, atrial, ventricular and tracheal tissues spiked with cocaine at low, medium and high concentration within a linear range of the calibration curve. Deviation of each cocaine concentration should be within ± 10 % of the spiked values. The coefficient of variance (CV) at each concentration of direct injection should be less than 5% and the extraction should be less than 10% (Table 5-8).

4.8 Extraction recovery

The extraction recovery was determined by comparing the peak area obtained from the extracted samples containing a known amount of cocaine with those obtained from a direct injection of the same concentration. The percentage recovery was calculated as follows:

$$\frac{\text{Peak area obtained from an extracted sample} \times 100}{\text{Peak area obtained from a direct injection}}$$

The extraction recovery of cocaine from plasma, atrial, ventricular and tracheal tissue homogenates were demonstrated in Table 5-8.

5. **Data analysis**

The concentrations of cocaine in plasma, cardiac, and tracheal tissues were expressed as means ± standard error of means ($\bar{x} \pm$S.E.M). All data were analyzed using unpaired Student *t*-test. Difference was considered significant if p value < 0.05.

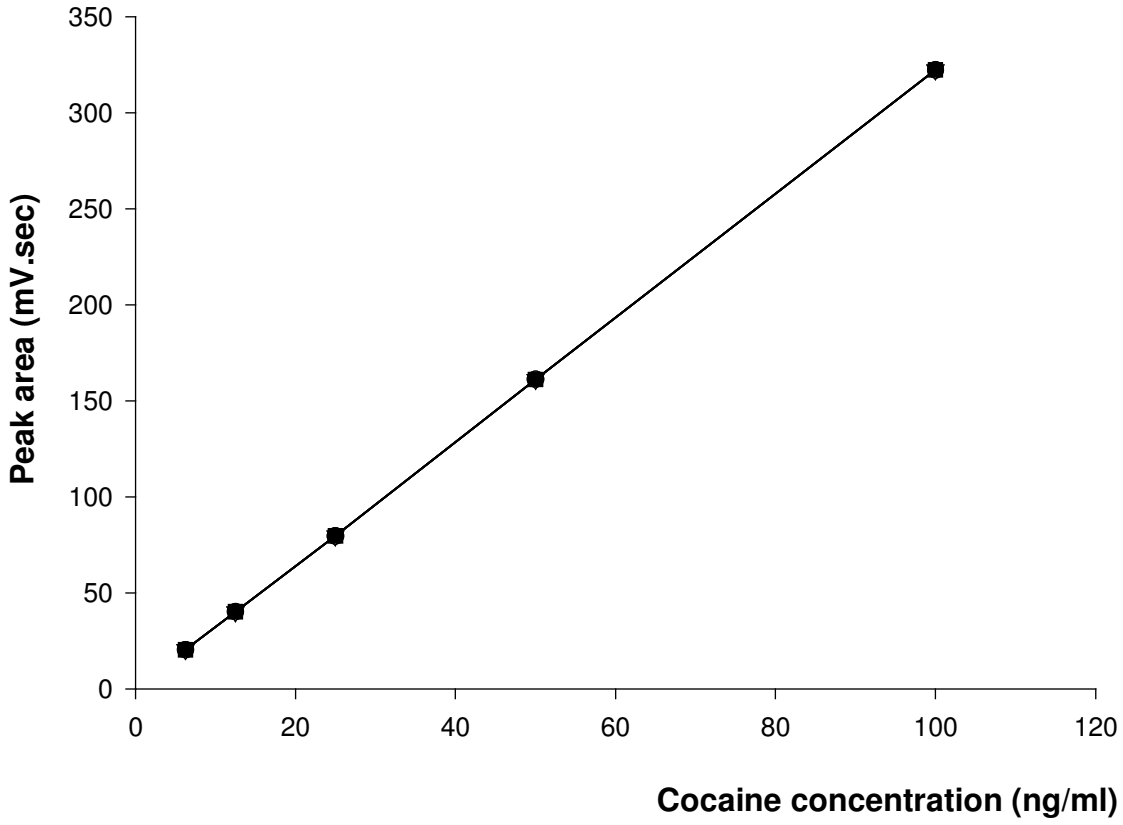


Figure 13 Calibration curve of cocaine in *plasma*, correlation coefficient (r) = 0.999
Peak Area =3.188(concentration)-0.900

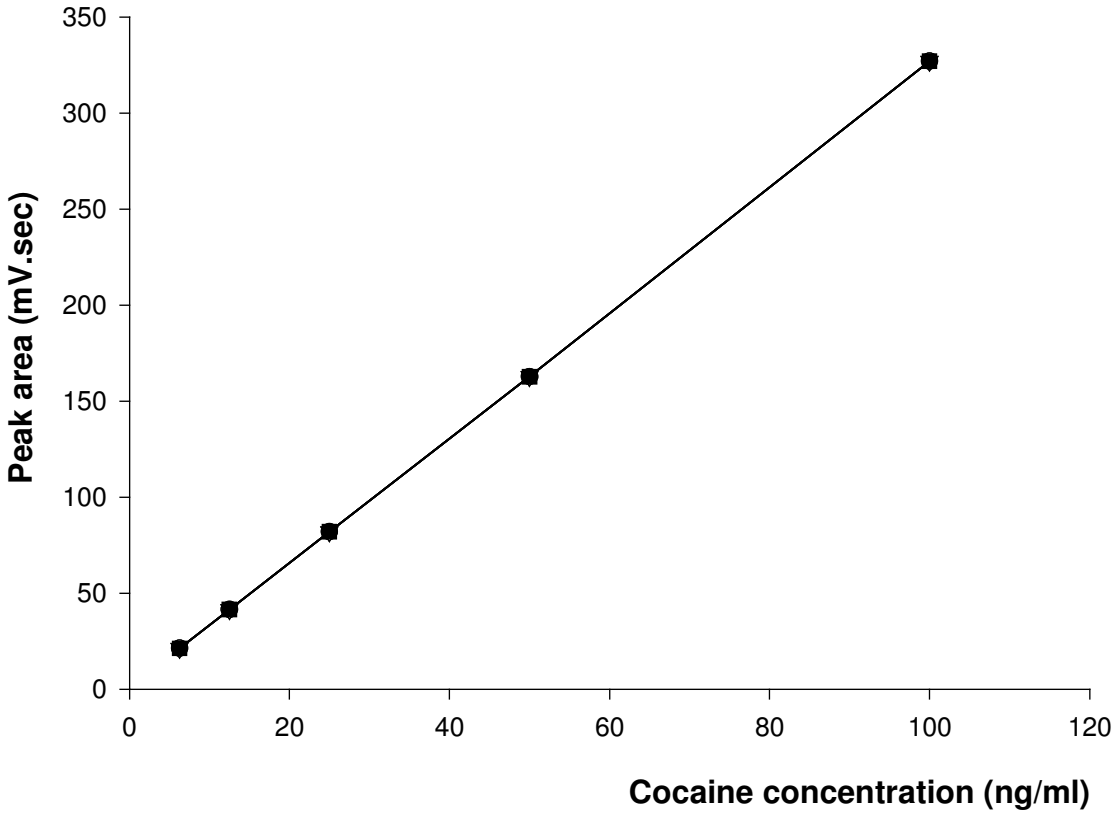


Figure 14 Calibration curve of cocaine in *atria*, correlation coefficient (r) = 0.999
Peak Area =3.270(concentration) + 0.605

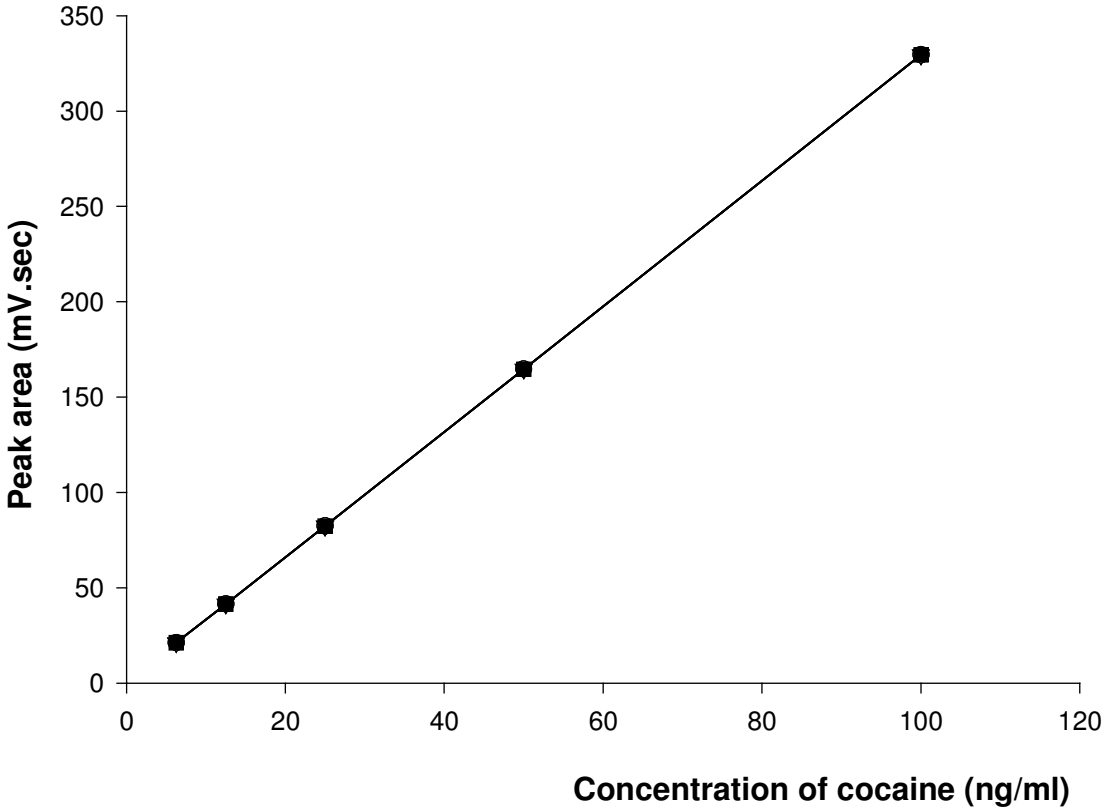


Figure 15 Calibration curve of cocaine in *ventricle*, correlation coefficient (r) = 0.999
Peak Area =3.279(concentration) + 1.175

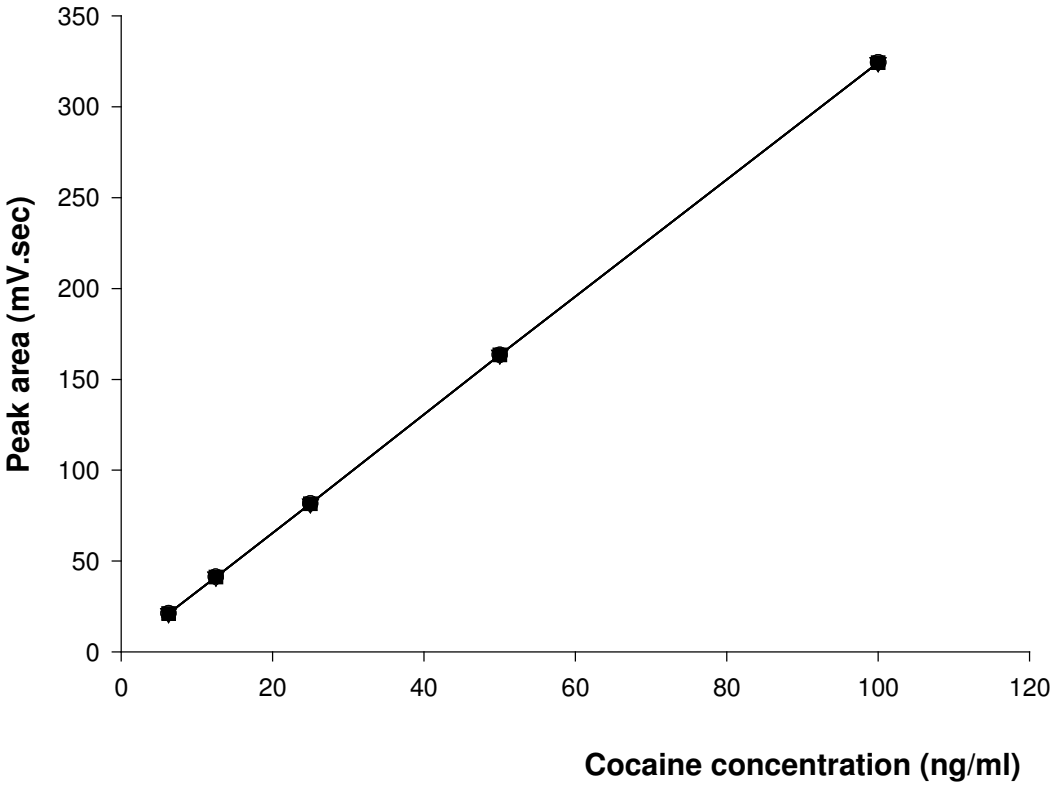


Figure 16 Calibration curve of cocaine in *trachea*, correlation coefficient (r) = 0.999
Peak Area =3.232(concentration) + 1.544

Table 5 The validation parameters of cocaine concentrations in *plasma*

Validation parameters	Plasma Concentration (ng/ml)				
	6.25	12.5	25	50	100
1. Precision 1.1 Intra-day CV (%)	3.39	2.63	1.19	1.00	0.80
1.2 Inter-day CV (%)	4.13	2.18	1.22	1.5	1.78
2. Accuracy Recovery (%)	95.15	96.48	95.89	96.33	96.73
3. linearity of method					
3.1 r	0.999				
3.2 r^2	1.000				
4. LOD (ng/ml)	0.69				
5. LOQ (ng/ml)	2.10				

Table 6 The validation parameters of cocaine concentrations in *atria*

Validation parameters	Atrial Concentration (ng/ml)				
	6.25	12.5	25	50	100
1. Precision 1.1 Intra-day CV (%)	3.25	2.00	1.01	1.04	0.98
1.2 Inter-day CV (%)	4.09	3.37	1.56	0.68	0.84
2. Accuracy Recovery (%)	99.54	99.83	98.71	97.25	98.12
3. linearity of method					
3.1 r	1.000				
3.2 r^2	1.000				
4. LOD (ng/ml)	0.70(17.5 ng/g)				
5. LOQ (ng/ml)	2.11(52.75 ng/g)				

Table 7 The validation parameters of cocaine concentrations in *ventricle*

Validation parameters	Ventricular Concentration (ng/ml)				
	6.25	12.5	25	50	100
1. Precision 1.1 Intra-day CV (%)	4.33	2.17	1.04	1.24	0.84
1.2 Inter-day CV (%)	3.18	3.23	1.45	1.18	1.58
2. Accuracy Recovery (%)	99.18	99.77	99.30	98.42	98.95
3. linearity of method					
3.1 r	0.999				
3.2 r^2	1.000				
4. LOD (ng/ml)	0.76 (3.8 ng/g)				
5. LOQ (ng/ml)	2.32 (11.6 ng/g)				

Table 8 The validation parameters of cocaine concentrations in *trachea*

Validation parameters	Tracheal Concentration (ng/ml)				
	6.25	12.5	25	50	100
1. Precision 1.1 Intra-day CV (%)	3.21	3.01	1.35	1.24	0.80
1.2 Inter-day CV (%)	3.14	3.23	2.1	1.14	0.78
2. Accuracy Recovery (%)	99.07	99.20	98.29	97.73	97.41
3. linearity of method					
3.1 r	1.000				
3.2 r^2	1.000				
4. LOD (ng/ml)	0.69 (0.80 ng/g)				
5. LOQ (ng/ml)	2.10 (2.4 ng/g)				