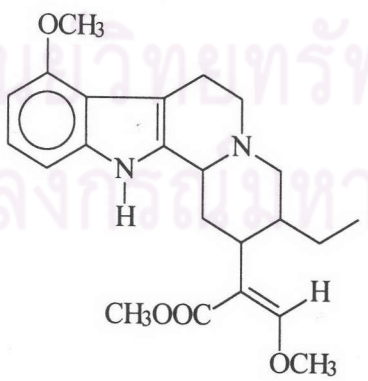
Chapter III

Results

A: Preparation of Mitragynine

The crude alkaloidal extract contained several alkaloids. After first isolation with column chromatography, the fraction containing mitragynine also contained at least 3 other alkaloids. This fraction was subjected to subsequent column chromatographic isolation using different solvent system to yield single component fraction with yellowish solution.

After the complete evaporation, this fraction, so-called substance A, produced a pale yellowish amorphous solid, that is soluble in diethyl ether, ethyl acetate, chloroform and methanol. This substance is identical in hRf values, IR (figure 8, 9) and 1H-NMR spectra (figure 10, 11) with authentic sample of mitragynine obtained from *Mitragyna speciosa* Korth.. The identification was also confirmed by ^{13}C-NMR spectrum (figure 12). Therefore, it was concluded that substance A was mitragynine with a chemical structure shown below.



Molecular weight = 398

Figure 7. Structure of mitragynine

B: Pharmacological Experiments

1. Studies of Analgesic Activity:

1.1 Hot plate test

Analgesic effect of mitragynine was observed as an increase in duration during which the mice could tolerate standing on a hot plate, which produced pain by thermal stimuli. Mitragynine at the dose of 30, 45 and 60 mg/kg body weight injected intraperitoneally 30 minutes before placing the mice on the hot plate significantly prolonged latency of nociceptive response in a dose dependent manner (figure 13). The maximum possible effect (% MPE) was shown in figure 14.

In contrast with morphine, antinociceptive effect of mitragynine was not reversed by the pretreatment of naloxone (figure 15). Thus, morphine at the dose of 10 mg/kg prolonged nociceptive latency in the hot plate test. Naloxone (0.1 mg/kg), given 15 min prior to morphine injection, abolished the antinociceptive response of the latter. The same dose of naloxone had no discernible effects on mitragynine induced antinociceptive action.

1.2 Writhing test

Mitragynine produced analgesic effect in writhing test as indicated by a decrease in number of time the mice stretched their bodies after intraperitoneally injection of acetic acid. Mitragynine ,at the dose of 15, 30, 45 and 60 mg/kg body weight, were injected intraperitoneally 30 minutes before injecting 0.6% acetic acid solution. Reduction of number of stretch was observed at the doses of 30, 45 and 60 mg/kg (figure 16) The antinociceptive effect of mitragynine is shown in figure 17.

1.3 Formalin test

The injection of 20 μl of 1 % formalin in normal saline solution into a hind paw caused pain which could be divided into 2 phases, early phase and late phase. The pain response was measured as the duration the mice used in licking their hind paw after injection, presumably in attempt to relief the pain caused by the injection. Mitragynine was injected 30 minutes prior the test at the dose of 15, 30, 45, and 60 mg/kg body weight. Control group was injected with 0.5 % CMC. Mitragynine produced changes in pain response of the mice in both phase. The pattern of antinociceptive effect of mitragynine is dose dependent manner. Mitragynine at the dose of 30, 45 and 60 mg/kg showed significant decrease in licking time in both phases (figure 18). As well as intraperitoneal injection, mitragynine given orally showed the same effect (figure 19).

2. Locomotor activity

At the dose of 15 and 30 mg/kg body weight injected intraperitoneally, mitragynine markedly increased the locomotor activity of the mice as expressed in term of total distance and number of movement . The response was dose dependent. However, at the dose of 60 mg/kg body weight the locomotor activity became decreased when compared with the dose of 30 mg/kg body weight as shown in figure 20. Figure 21 showed that haloperidol, a dopamine 2 receptor antagonist, decrease locomotor activity of mice. However at the dose of 0.1 mg/kg, haloperidol which has no significant effect on locomotor activity (figure 21), can antagonize mitragynine induced increase locomotor activity. (figure 22).

3. Core body temperature

This study indicated mitragynine induced hypothermia in mice. Mitragynine, at the dose of 30 and 60 mg/kg, significantly decreased body temperature. The hypothermia can be observed 30 min after injection of

mitragynine. The decrease in core body temperature was a dose dependent manner. Mitragynine at the dose of 60 mg/kg body weight significantly decrease core body temperature more than 1 degree Celsius (figure 23).

4. Rotational behavior (Turning behavior)

6-Hydroxydopamine (6-OHDA) can be uptaken into dopaminergic neurons in substantia nigra resulting in the degeneration of the neurons by virtue of its strong oxidizing property. The final cascade is the hypersensitivity of the dopamine receptor in the receptive area, i.e. the striatum, causing imbalance of movement especially after receiving dopamine agonist or dopamine releasing agent. The induced rotational behavior can be differentiate between dopamine agonist or dopamine releasing agent. The dopamine agonist induced contralateral rotation whereas dopamine releasing agent causes the rat to rotate ipsilaterally.

In this experiment, 6-OHDA lesioned rat was injected with 0.1 mg/kg body weight of apomorphine ,subcuteneously, every 5 day until the constant rotation occurred. Mitragynine, injected intraperitoneally at the dose of 15 and 30 mg/kg body weight ,did not induced any rotation in lesioned. Additionally, pretreatment with mitragynine at the dose of 15 and 30 mg/kg body weight (30 minutes before 0.1 mg/kg apomorphine injection) did not show any significantly effect on apomorphine induced rotation (figure 24).

5. Effect of mitragynine on neurotransmitter levels; a microdialysis study.

The content of endogenous amines were determined by using HPLC isocratic separation and electrochemical detector with varying pH and composition of the mobile phase. Figure 25 showed chromatogram of biogenic amines and some metabolites. The HPLC system used in this study succeeded in separating and identifying the mixed endogenous amines in the sample as well as in the dialysate. The chromatogram of brain dialysate showed that the

dialysate contained detectable neurotransmitters and some metabolic products of neurotransmitters (figure 26) i.e. DOPAC, HVA and HIAA. The former two are the metabolic products of dopamine and the other is that of serotonin. The standard curves of DOPAC, HVA and HIAA were determined (figure 27, 28 and 29 respectively). Mitragynine at the dose of 15 and 30 mg/kg body weight given intraperitoneally did not produce any discoverable effect on the levels of these neurotransmitters, although some increase, but not significantly, was observed in some cases (figure 30, 31 and 32).

6. Dopamine D2 Receptor binding assay

Mitragynine, yohimbine, bromocriptine, and sulpiride were used as test substances. All of this substances can bind competitively to dopamine 2 (D2) receptor as shown by a decrease in percent binding of spiperone to D2 receptor. Mitragynine, at the concentration of 10^{-5} and 10^{-4} M replaced the binding of spiperone by 40 and 100 % respectively. In comparison, the control antagonists produced competition as follows: yohimbine (25% at 10^{-6}), sulpiride and bromocriptine (both > 50% at 10^{-7}) (figure 33).

7. Head twitch and head weaving.

5-methoxy-N,N-dimethyltryptamine (5-MeODMT) is a non selective serotonin agonist. It induces behavioral changes when given to the experiment animal depending on type of animals. The behavioral changes that occur is called serotonin syndrome such as head weaving and head twitch in mice.(Grahame-Smith, 1971) Mitragynine ,when given to mice, did not induced serotonin syndrome neither head weaving nor head twitch. Mitragynine at all doses also did not show any inhibition of 5-MeODMT induced head weaving (figure 34). In the other hand, head twitch induced by 5-MeODMT was inhibited when

pretreated with mitragynine in dose dependent manner (figure 35). Mitragynine at the dose of 20 and 30 mg/kg showed significant inhibition of 5-MeODMT induced head twitch.

8. Effect on Single Neuron: a microiontophoretic study

Mitragynine, when applied to Purkinje cell using microiontophoretic method, did not show any effect on firing rate of Purkinje cell. On the other hand, GABA and serotonin inhibited the spontaneous firing of Purkinje cell (figure 36) and mitragynine did not alter the effect of GABA and serotonin on firing rate of Purkinje cell (figure 36).

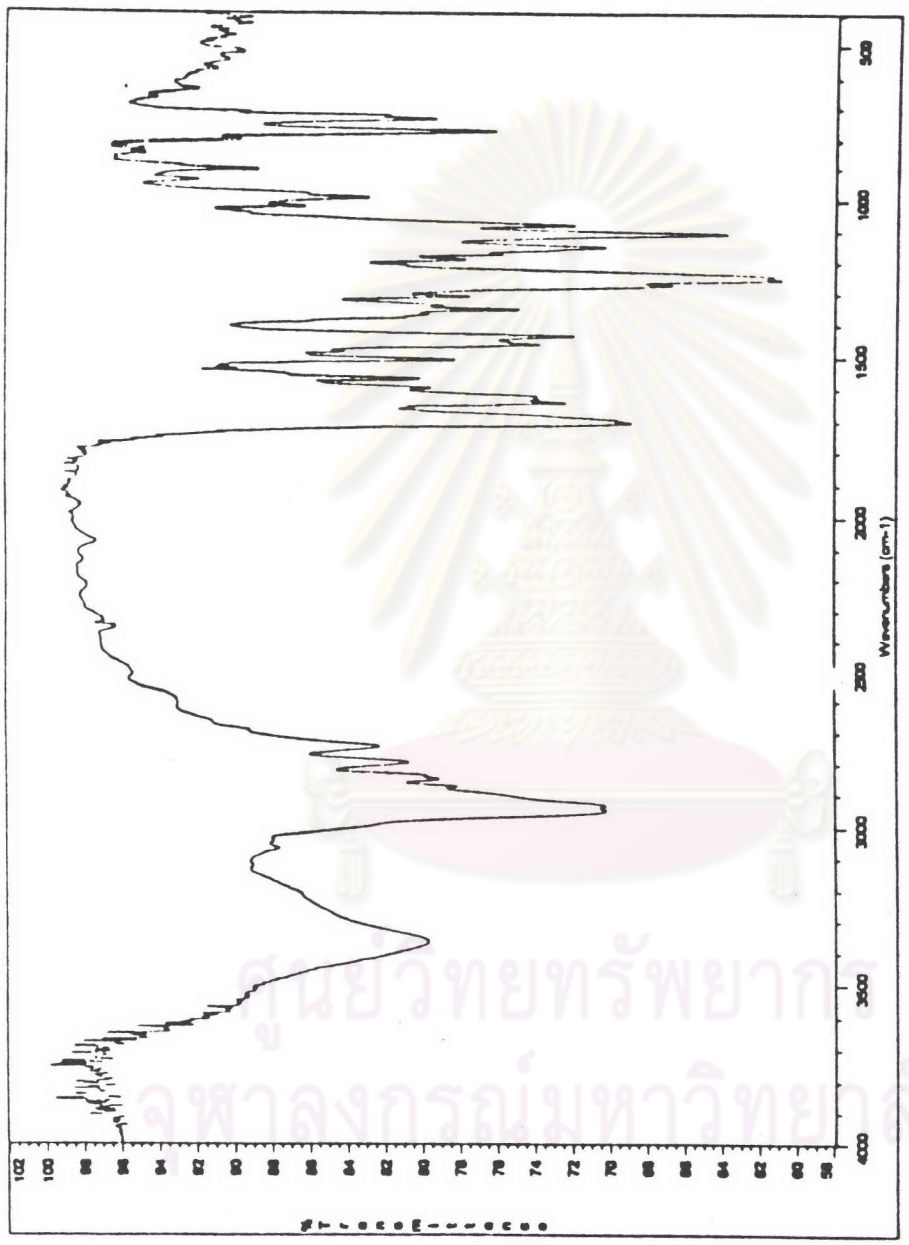


Figure 8 Infrared spectrum of substance A in KBr disk.

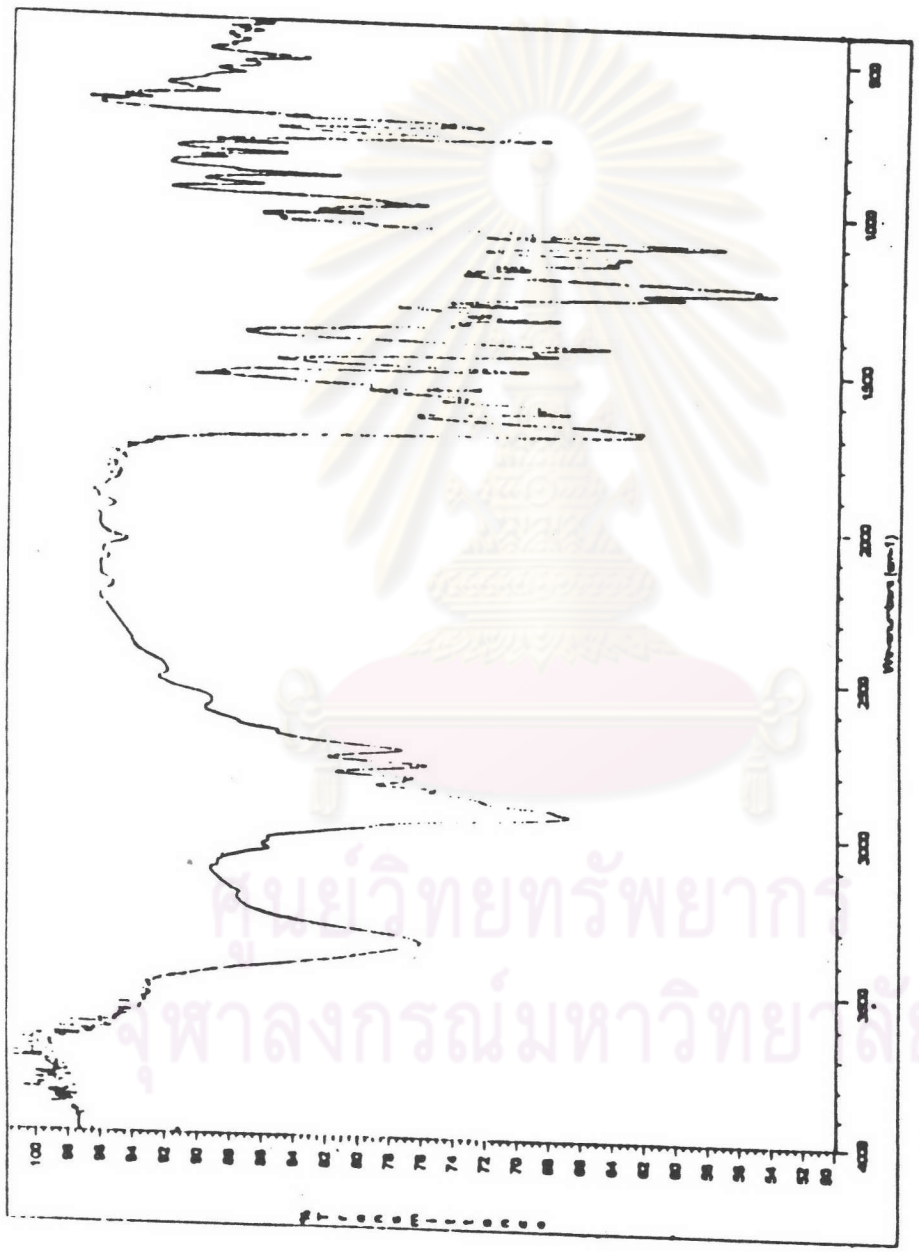


Figure 9. Infrared spectrum of mitragynine in KBr disk.

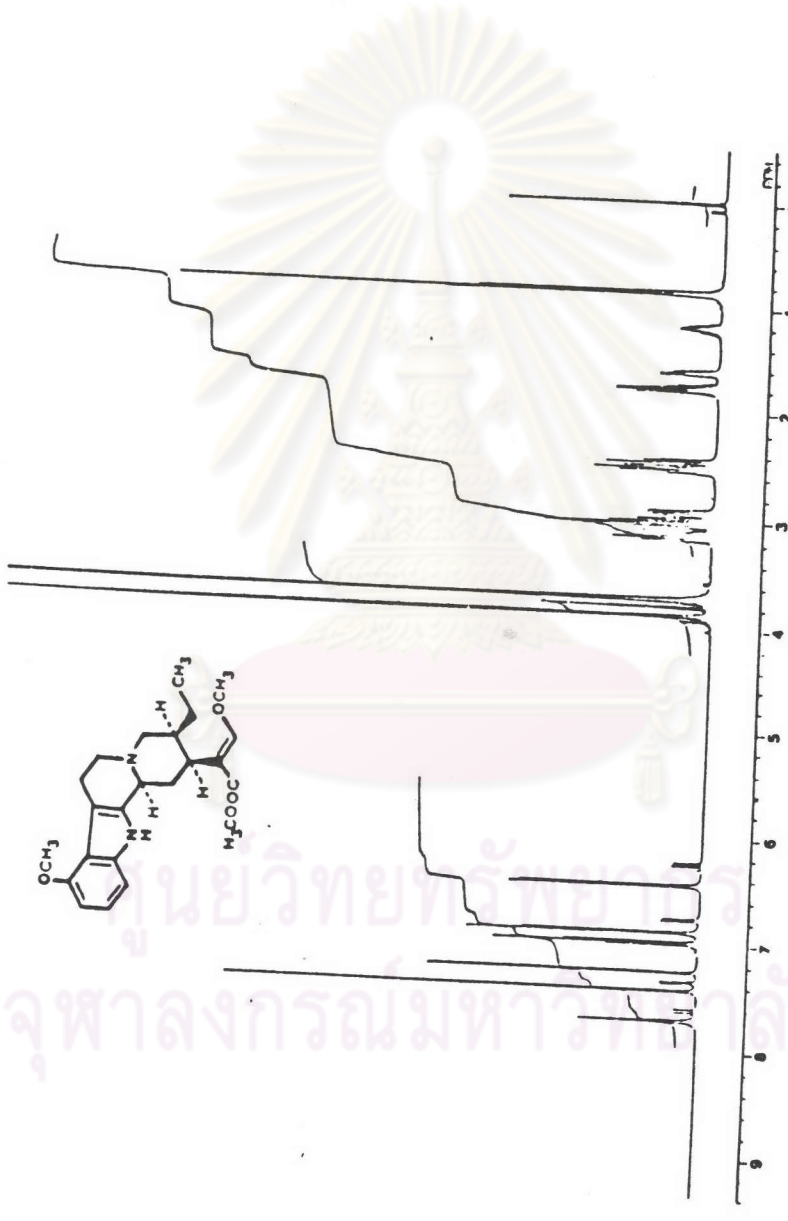


Figure 10. ^{1}H-NMR spectrum of mitragynine in $CDCl_3$

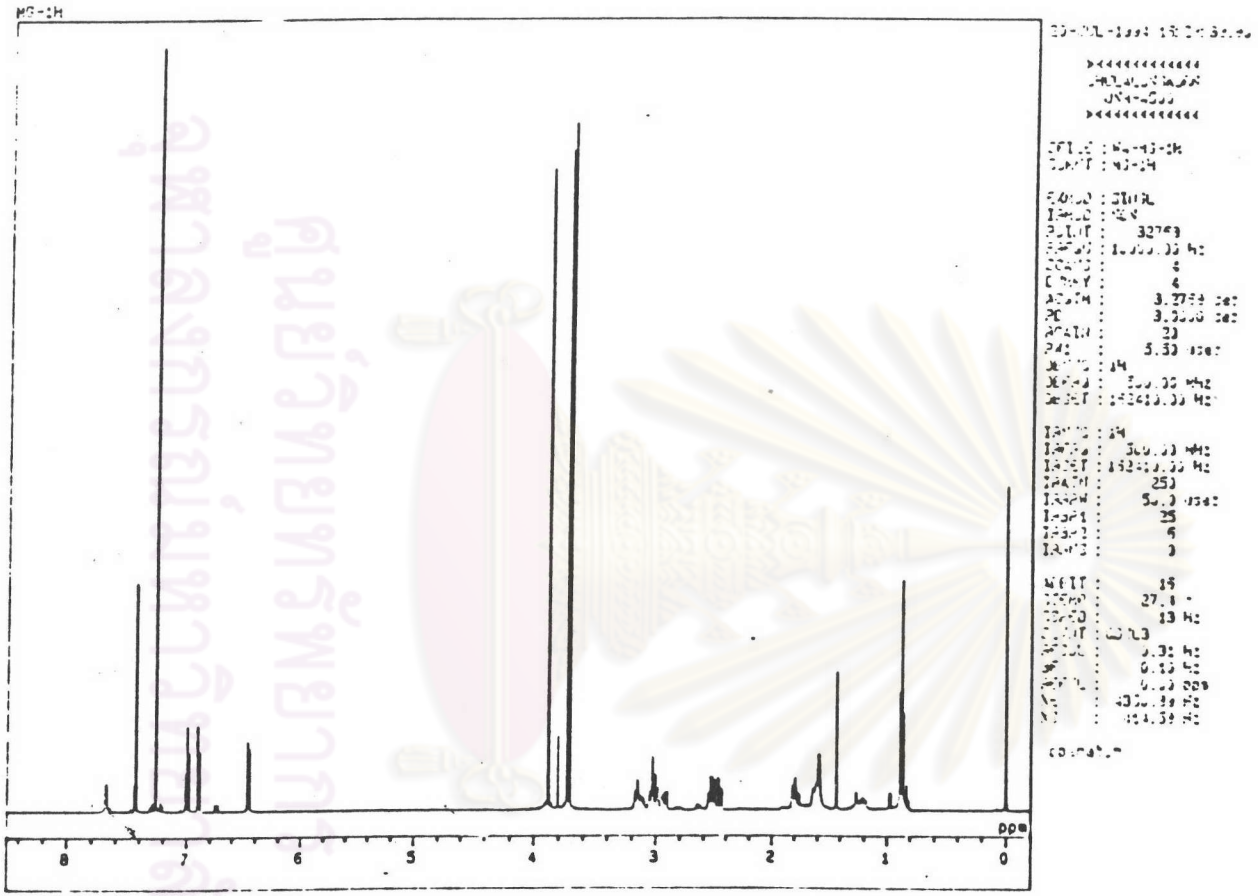


Figure 11. ^{1}H-NMR spectrum of substance A in $CDCl_3$

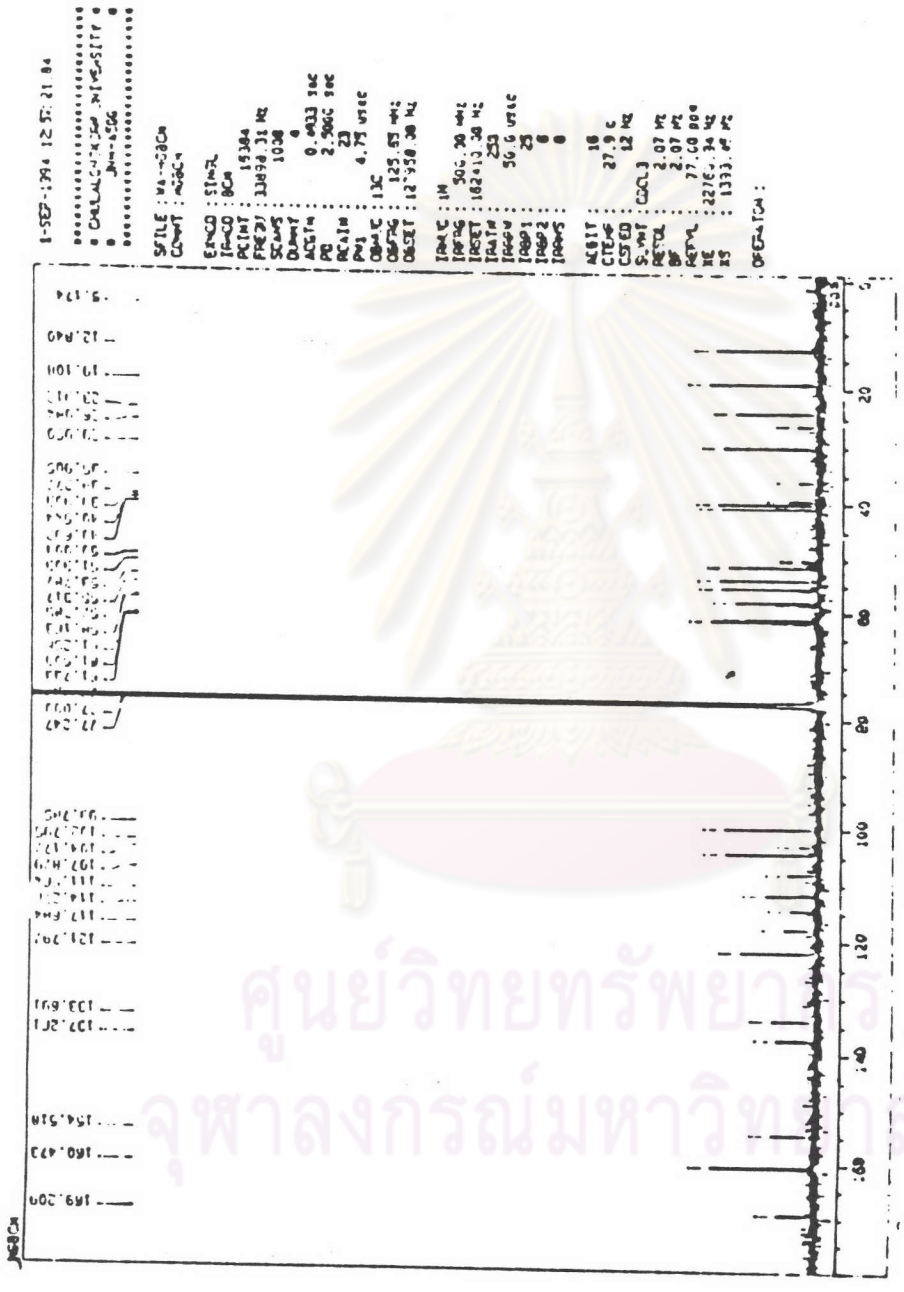


Figure 12. ^{13}C-NMR spectrum of substance A in $CDCl_3$

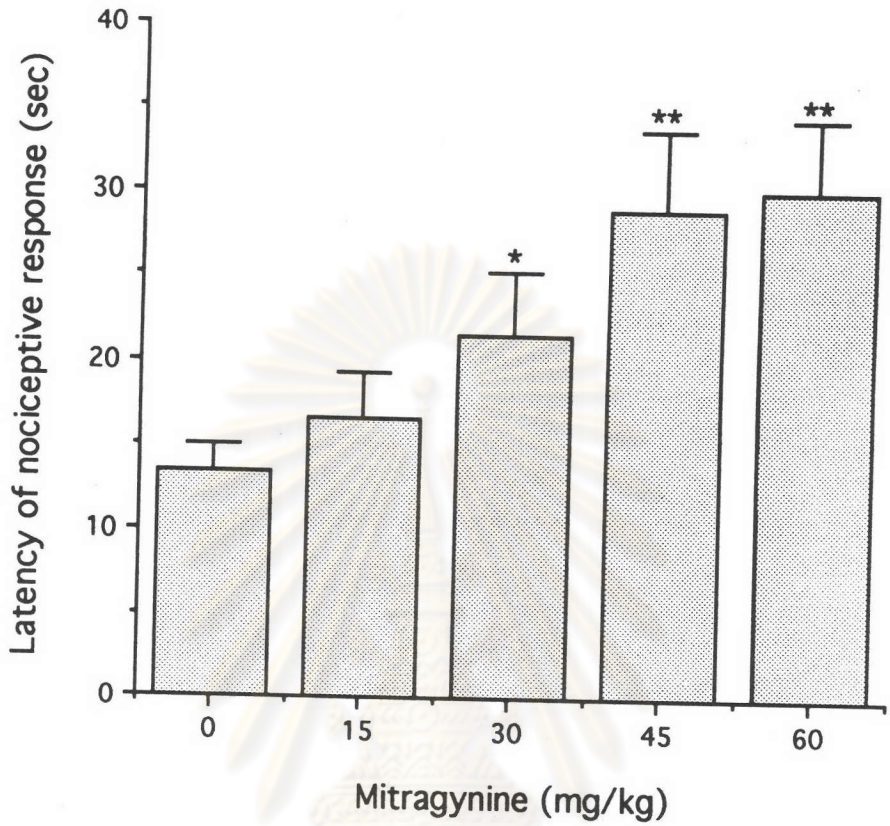


Figure 13. Effect of mitragynine on latency of nociceptive response in mice using hot plate test. Mitragynine was injected intraperitoneally 30 minutes before testing. 0.5% CMC in NSS was used as a control. *p<0.05 and **p<0.005 compared with control (n=10)

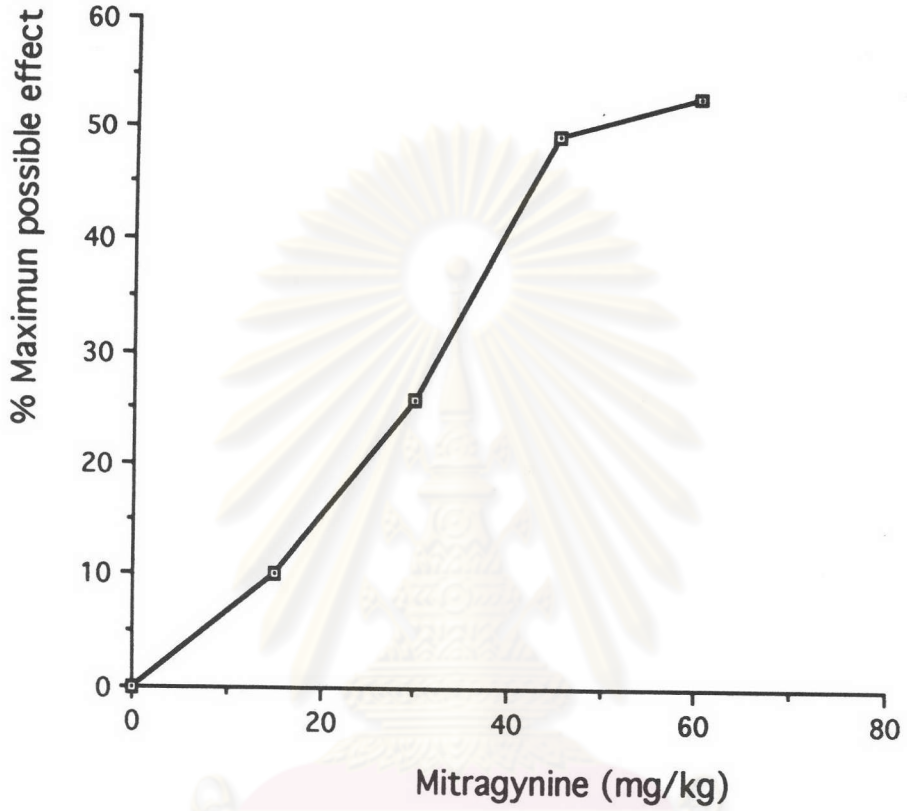


Figure 14. % Maximumpossible effect of mitragynine using hot plate test in mice. Mitragynine was injected intraperitoneally 30 min before testing. 0.5% CMC in NSS was used as a control(n=10).

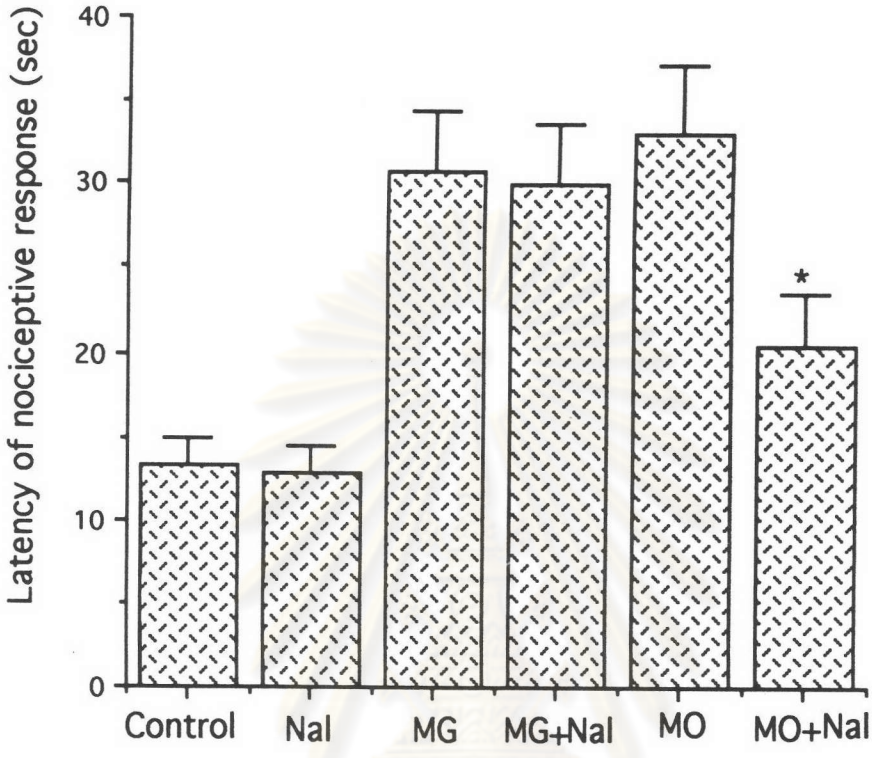


Figure 15. Effect of naloxone(Nal) on antinocicetive effects of morphine(MO) and mitragynine(MG). Naloxone was given 15 min. before morphine,10 mg/kg , and mitragynine, 60 mg/kg, intraperitoneally.*$p<0.05$ compared with Morphine(n=10)

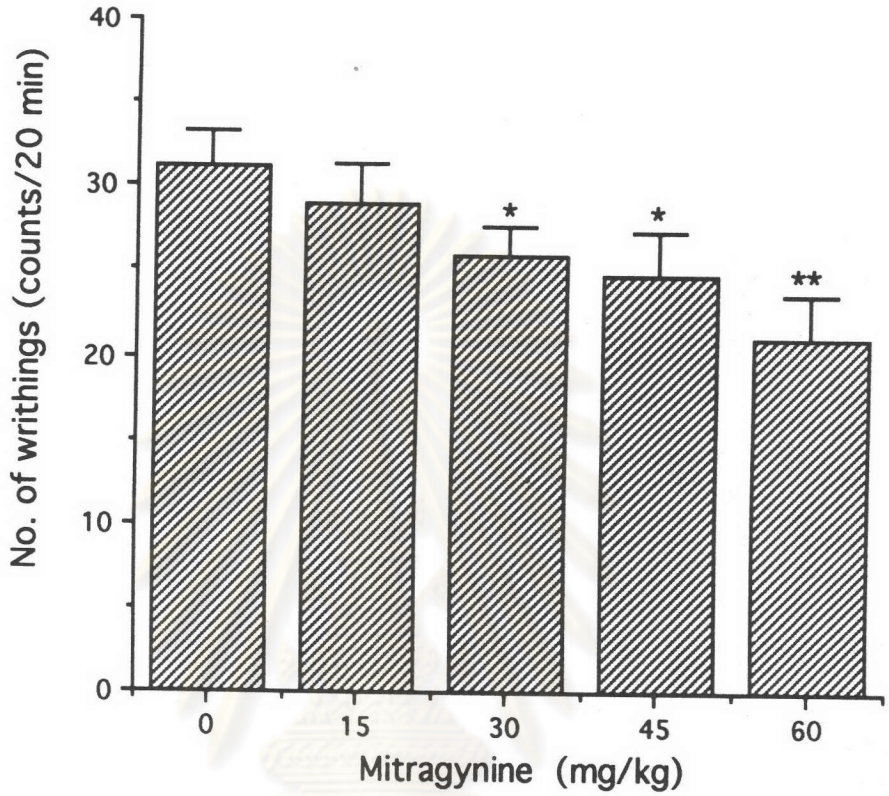


Figure 16. Effect of mitragynine on acetic acid - induced writhing in mice. Mitrgynine was injected intraperitoneally 30 min. before injection of 0.6% acetic acid solution intraperitoneally. 0.5% CMC was used as a control. *p<0.05 and **p<0.01 compared with control (n=10).

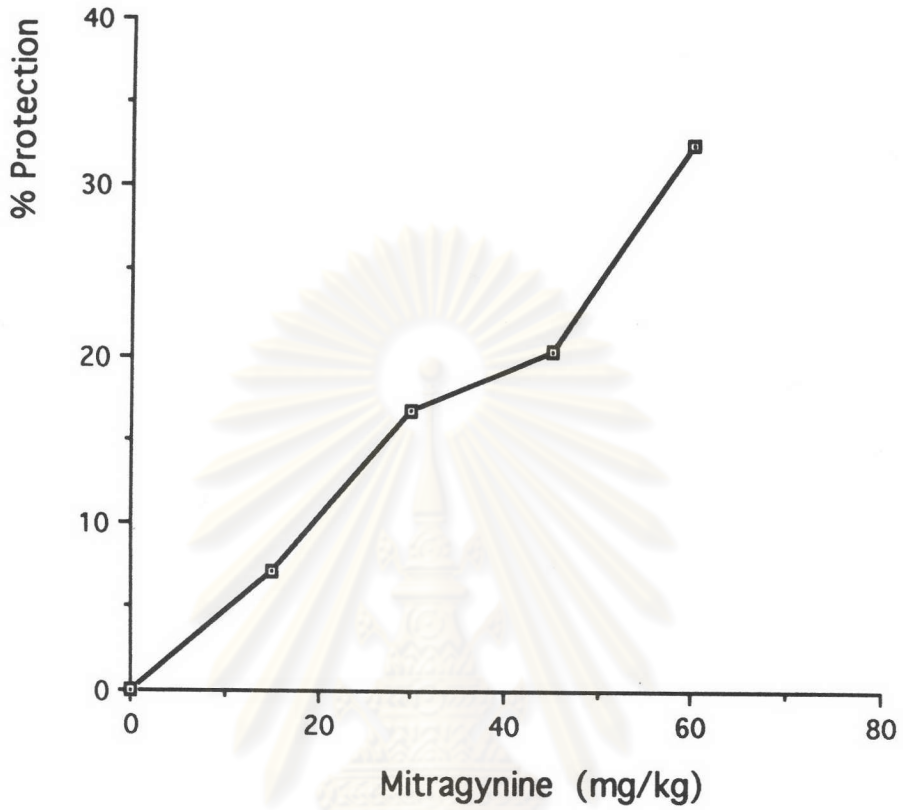


Figure 17. % Protection of mitragynine on acetic acid induced writhings. Mitragynine was injected intraperitoneally 30 min before injection of 0.6% acetic acid solution intraperitoneally (n=10).

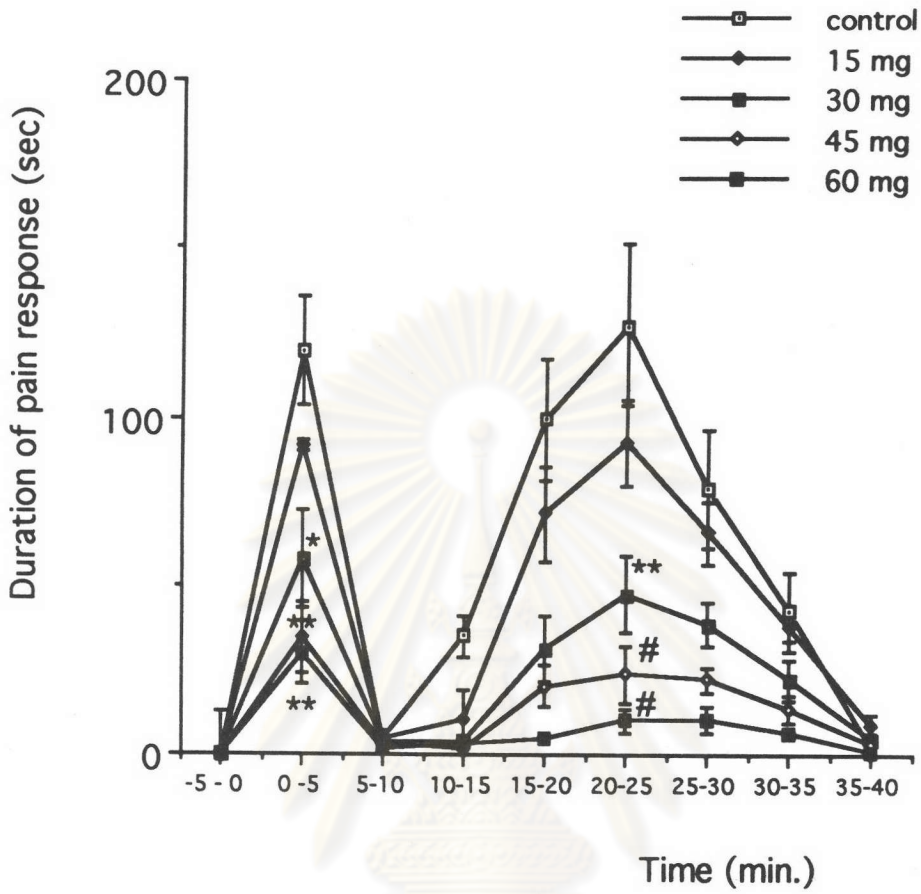


Figure 18. Effect of mitragynine on formalin induced nociception in mice. Mitragynine was given intraperitoneally 30 min. before formalin injection.*p<0.05 , **p<0.005 and #p<0.0005 compared with control at peak time(n=10)

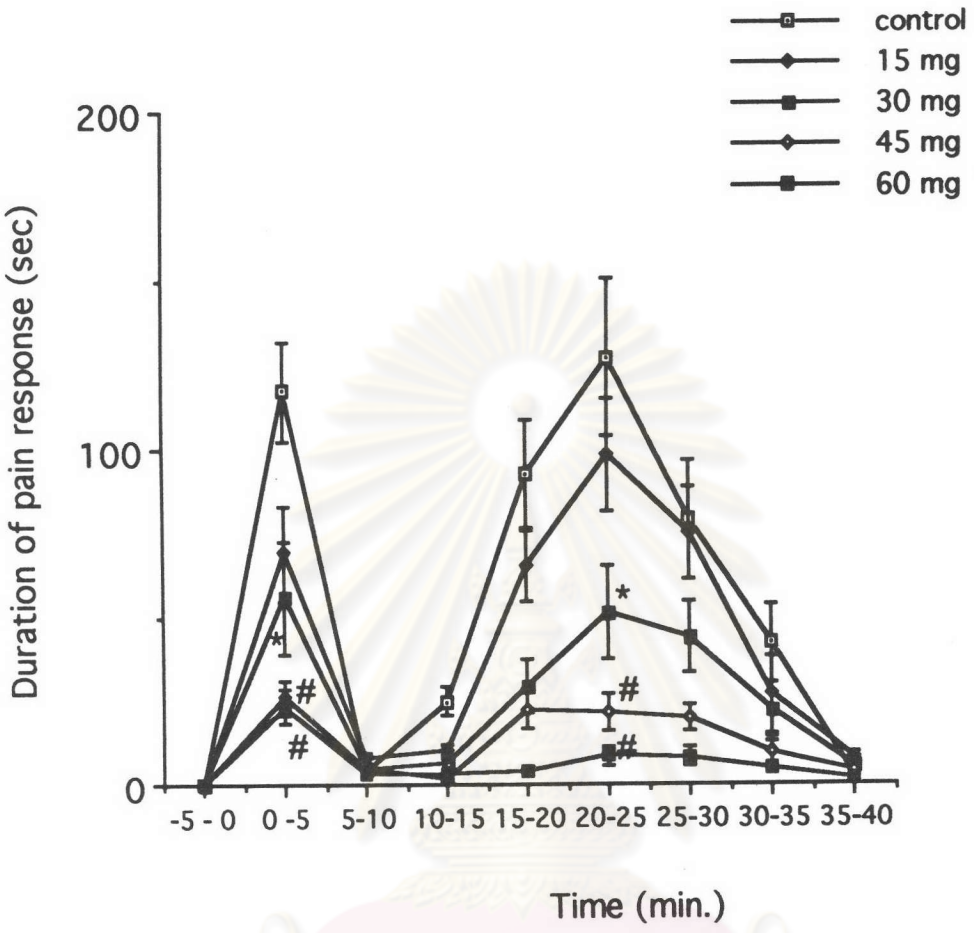


Figure 19. Effect of mitragynine on formalin induced nociception in mice. Mitragynine was given orally 30 min. before formalin injection. *p<0.01 and #p<0.0005 compared with control at peak time.(n=10)

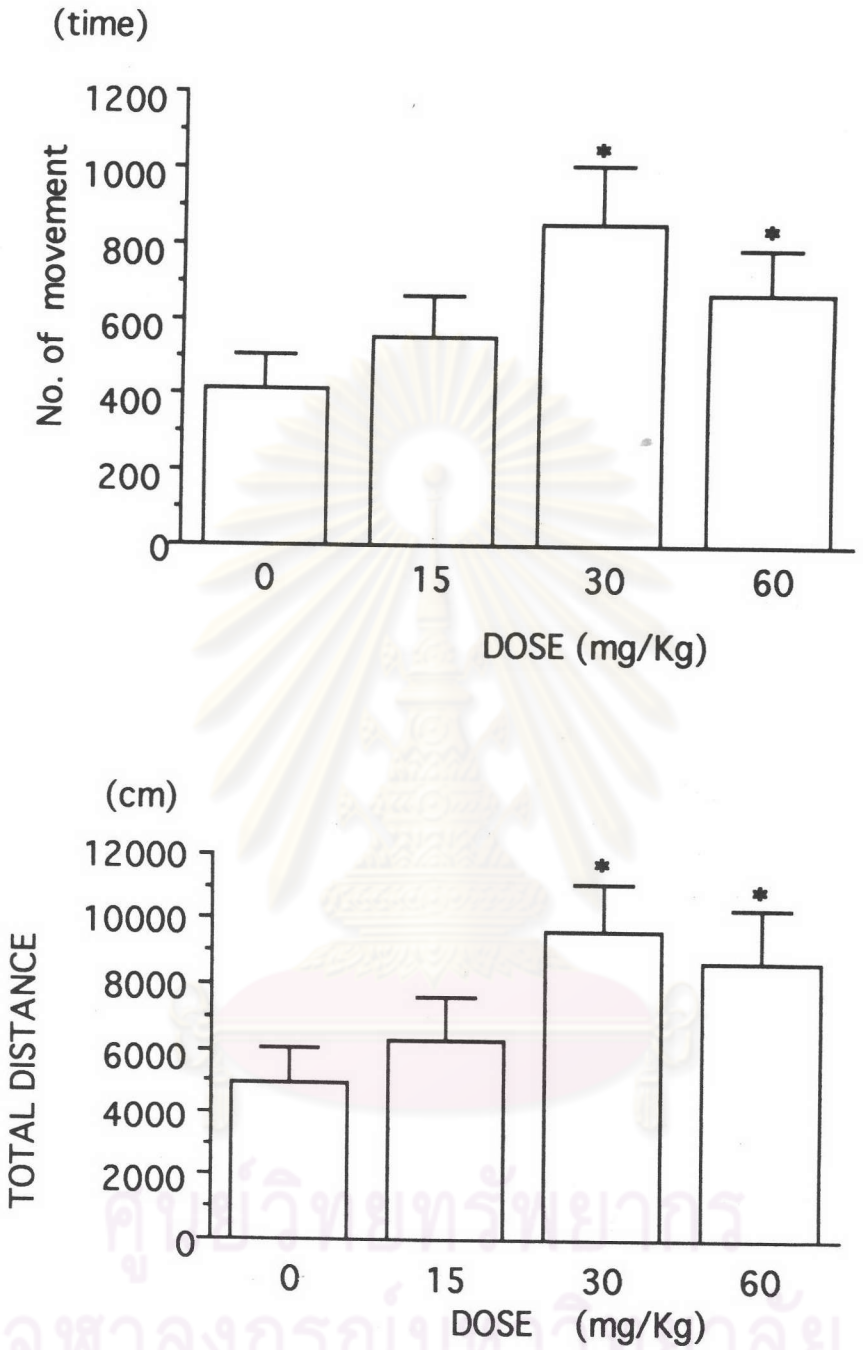


Figure 20. Effect of mitragynine on locomotor activity. Mitragynine was injected intraperitoneally. 0.5 % CMC was used as a control. *p<0.05 compared with control (n=9)

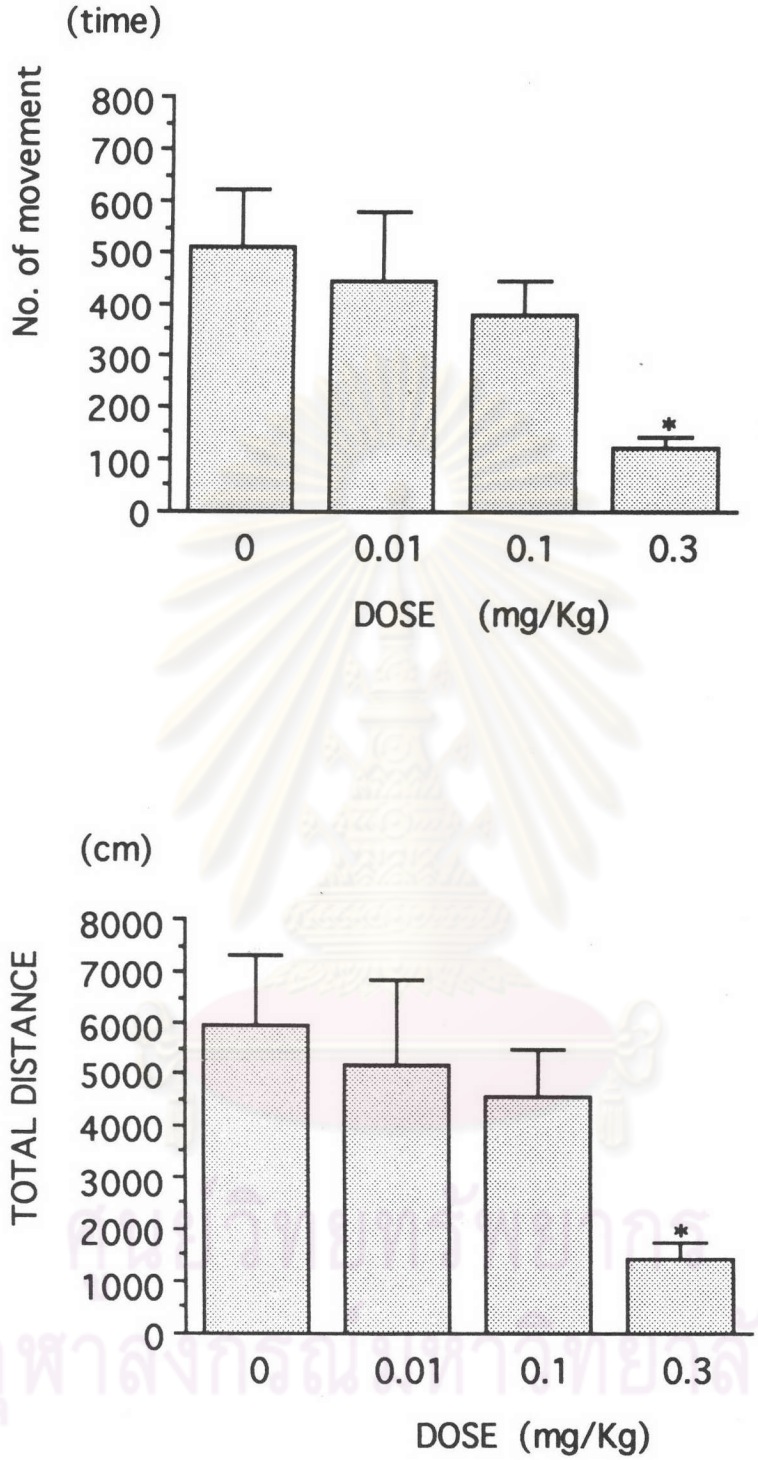


Figure 21. Effect of haloperidol on locomotor activity. Haloperidol was injected intraperitoneally using 0.9% NaCl as control.*p<0.05 compared with control(n=9).

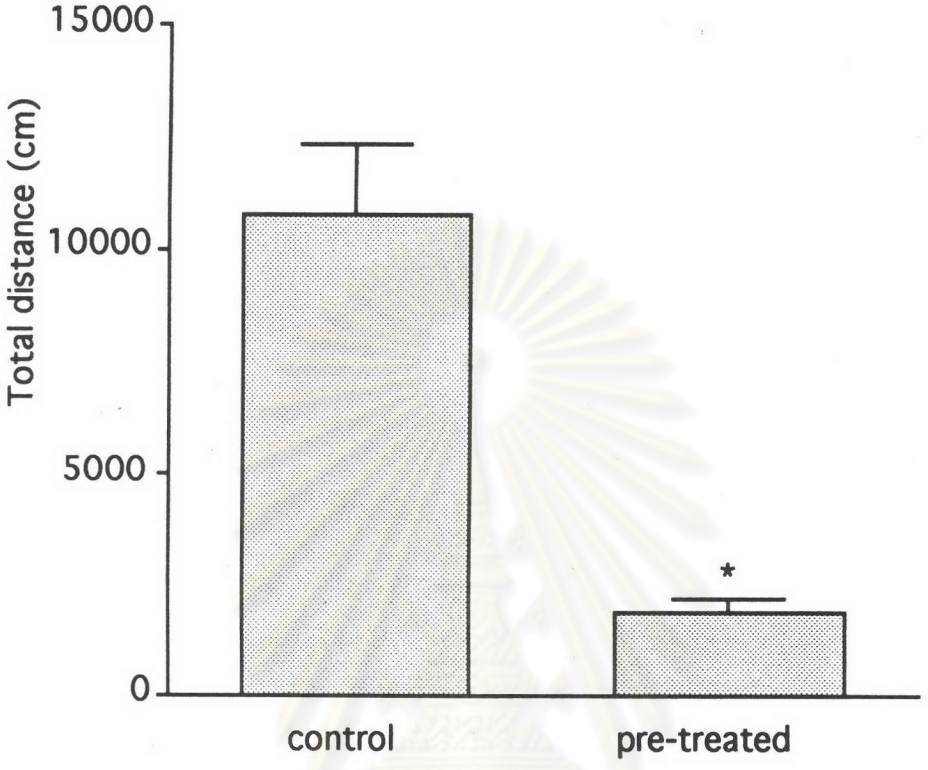


Figure 22. Effect of haloperidol on mitragynine induced increase in locomotor activity. Haloperidol, 0.1 mg/kg, was injected i.p. 30 min before mitragynine, 30 mg/kg i.p.. 0.9% NaCl was used as control. *p<0.01 compared with control (n=9).

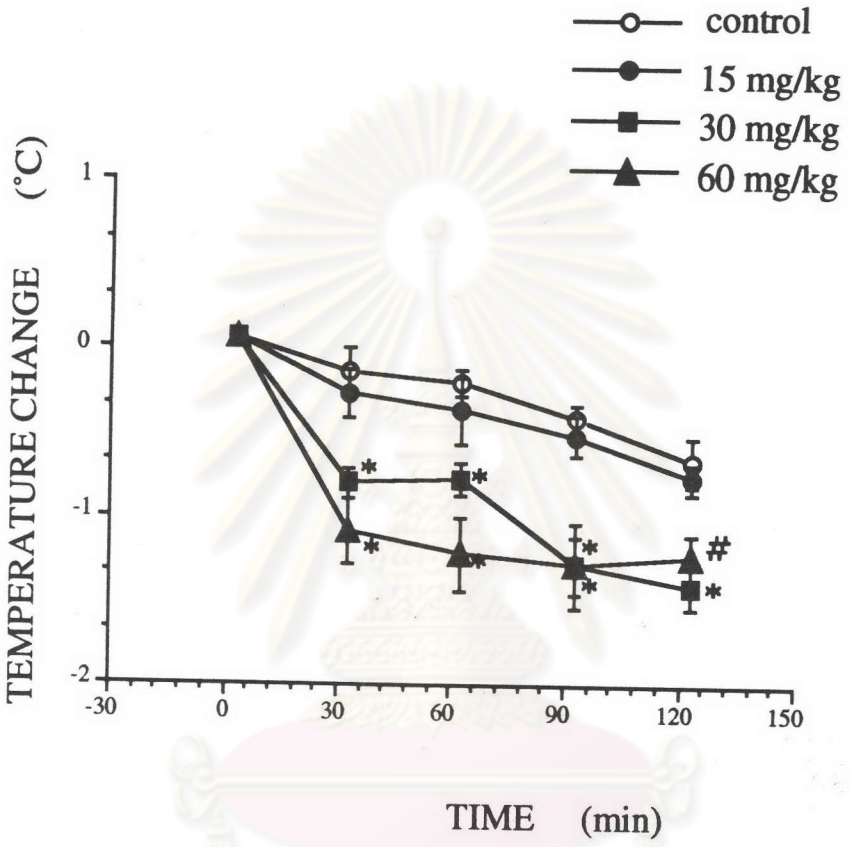


Figure 23. Effect of mitragynine on the core body temperature. Mitragynine was injected at the time 0. *p<0.005 and #p<0.01 compared with control (n=6).

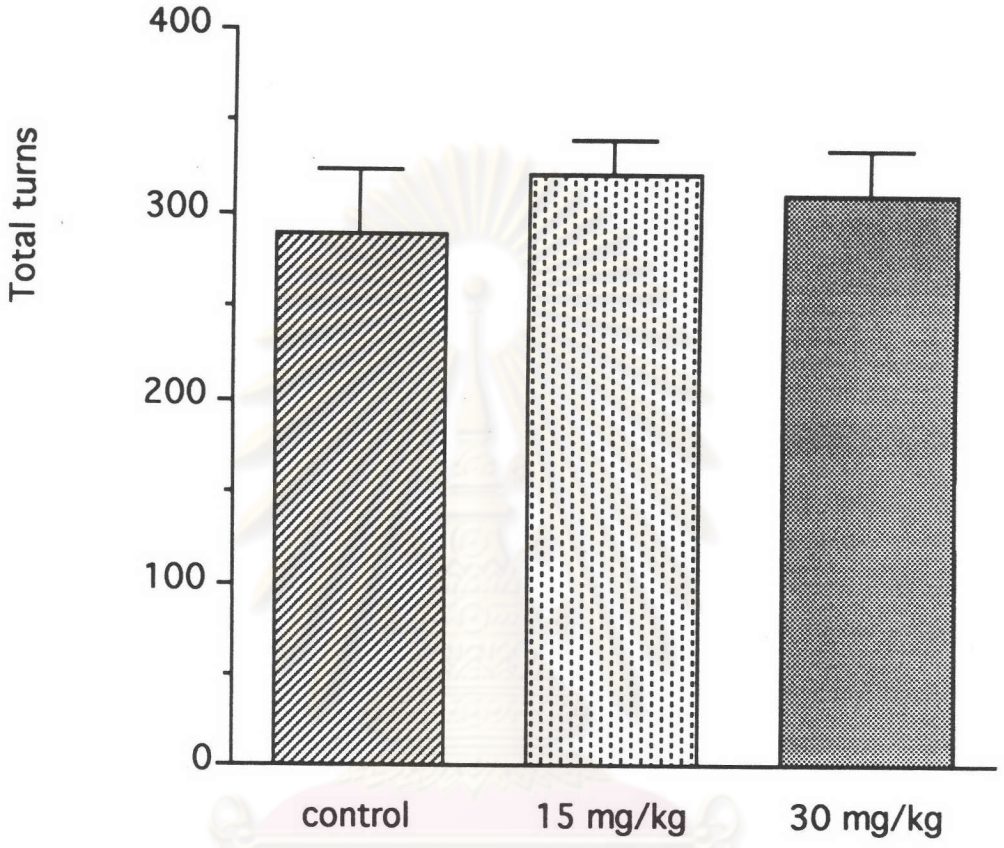


Figure 24. Effect of mitragynine on the 6-OHDA induced lesion. Mitragynine was injected 30 min before apomorphine 0.1 mg/kg,s.c. 0.5 % CMC in saline was used as control (n=6).

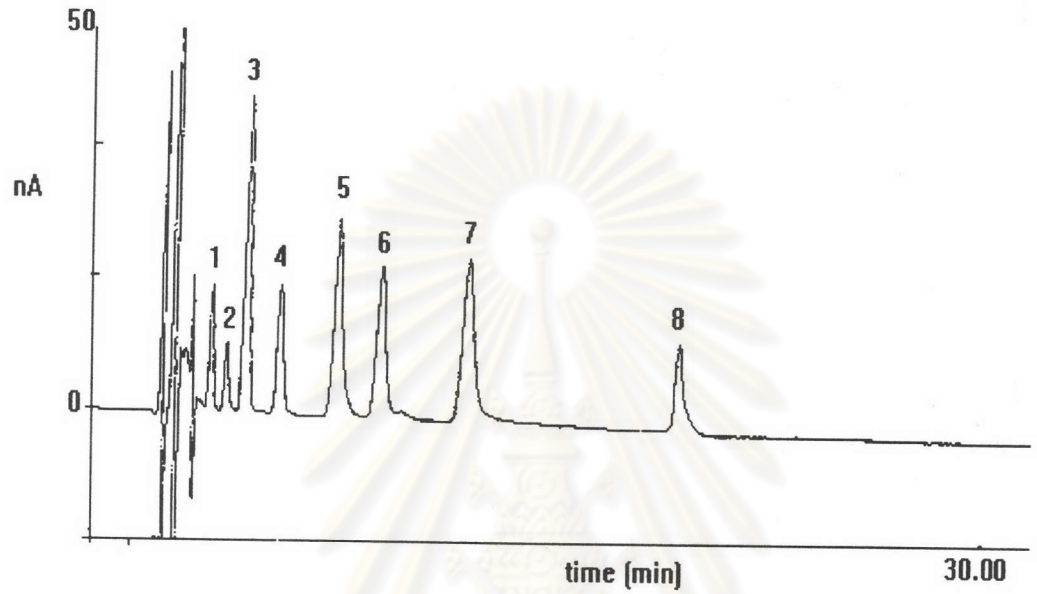


Figure 25. Chromatogram of biogenic amine and their metabolites detected by HPLC. The retention time (min) of each substance was shown in the parenthesis, 1=norepinephine(3.56), 2=epinephrine(4.48), 3=DOPAC(5.23), 4=dopamine(7.02), 5=HIAA(10.18),6=Isoproterenol used as internal standard(11.26),7=HVA(13.51), 8=serotonin(20.83).

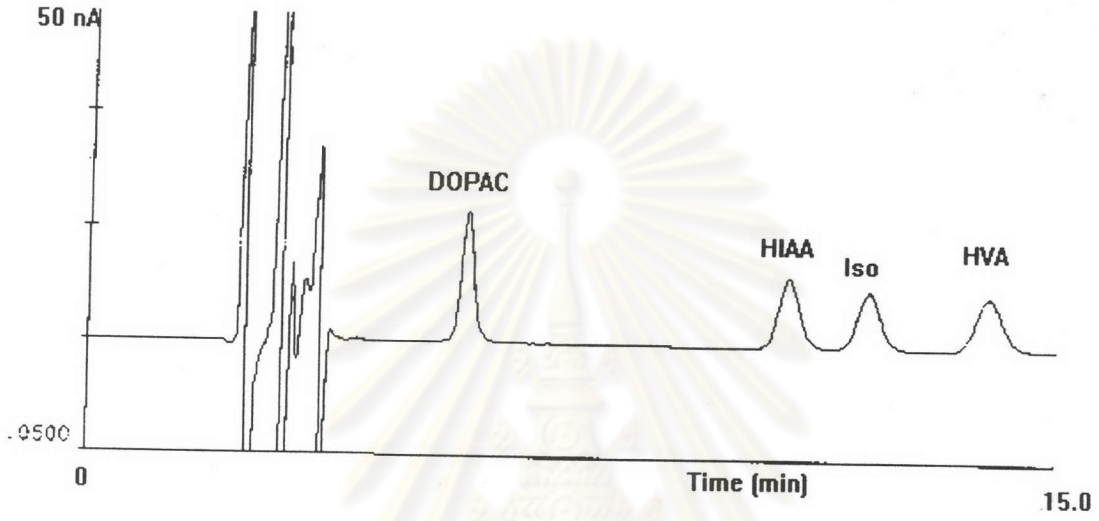


Figure 26. Chromatogram of brain dialysate detected by HPLC. Three metabolites were found and identified as DOPAC, HVA and HIAA using isoproterenol (Iso) as internal standard. The retention times of DOPAC, HIAA, Iso, and HVA were 6.01, 10.55 12.54 and 14.02 min respectively.

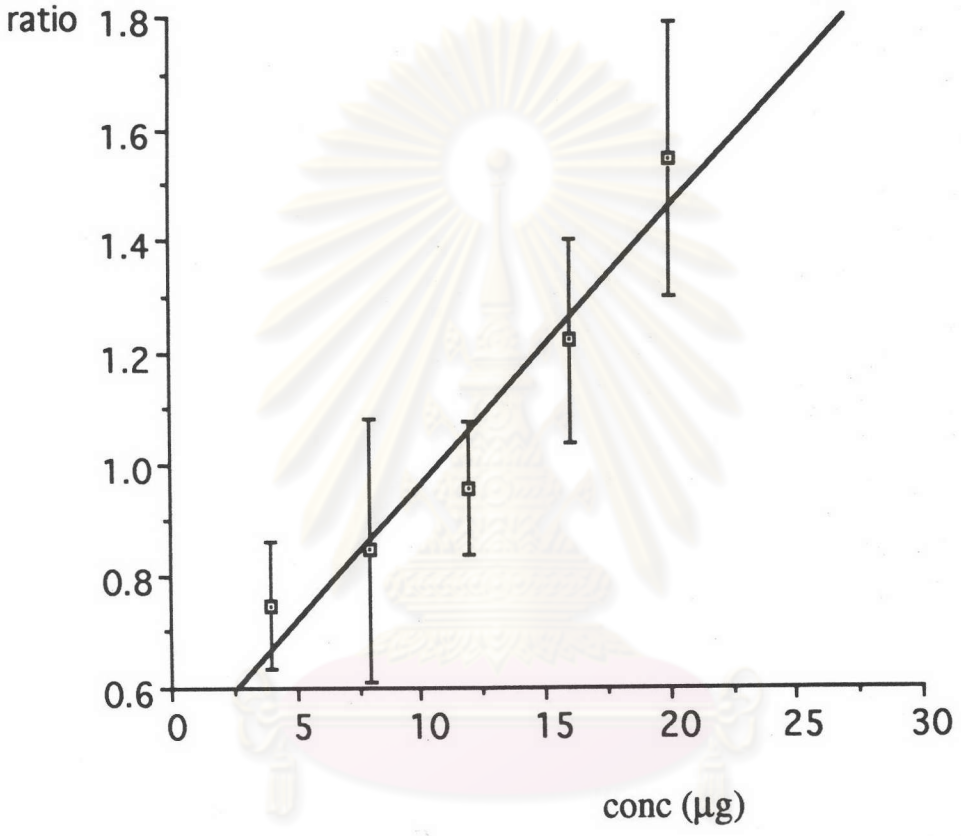


Figure 27. Standard curve of DOPAC . Compare between concentration of DOPAC and ratio of area of DOPAC and internal standard (Isoproterenol) (n=5).

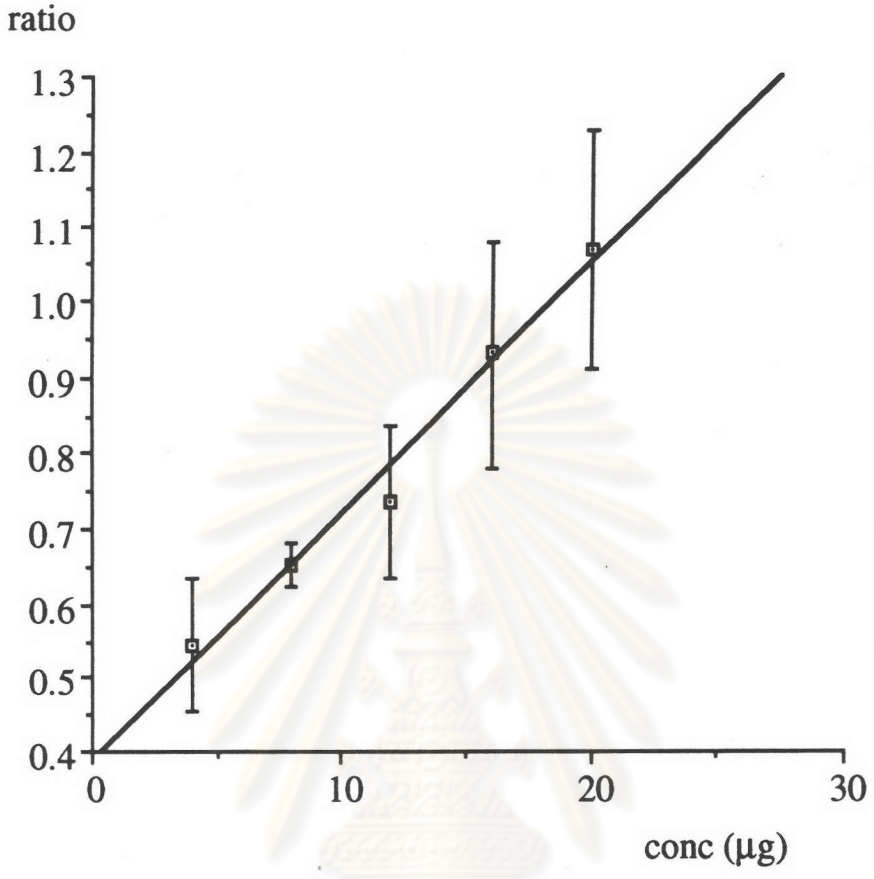


Figure 28. Standard curve of HVA. Compare between concentration of HVA and Ratio of are a of HVA and internal standard (Isoproterenol) (n=5)

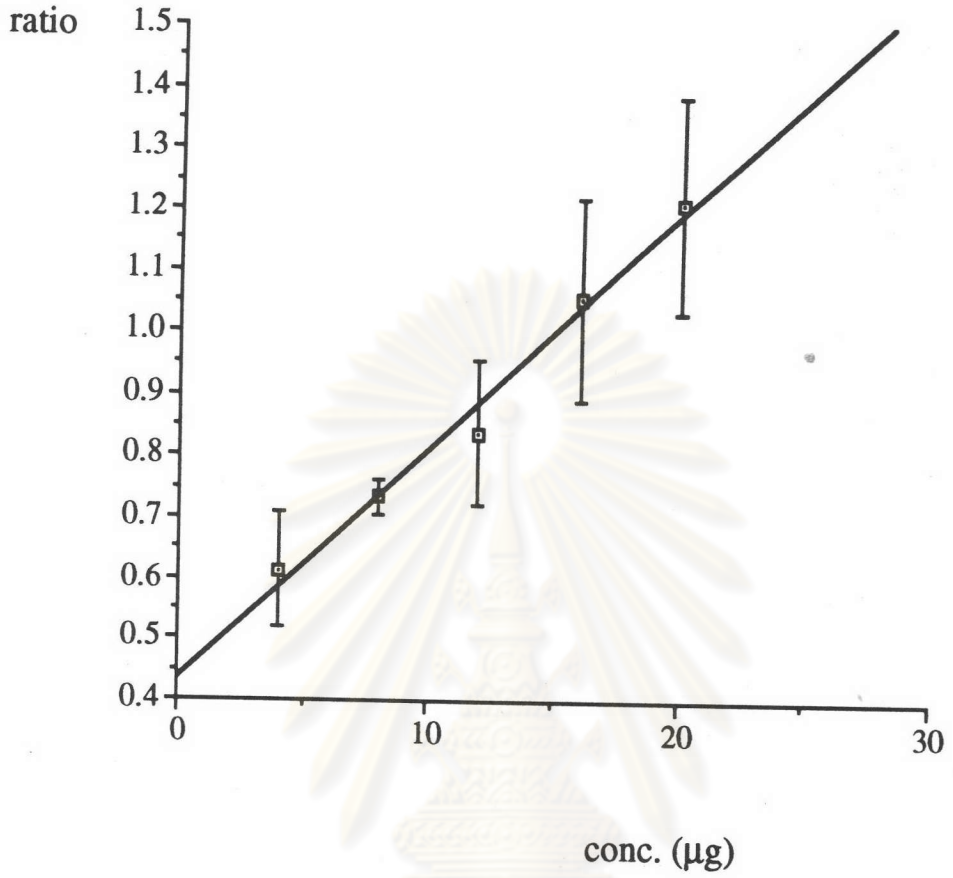


Figure 29. Standard curve of HIAA. Compare between concentration of HIAA and ratio of area of HIAA and internal standard(Isoproterenol) (n=5)

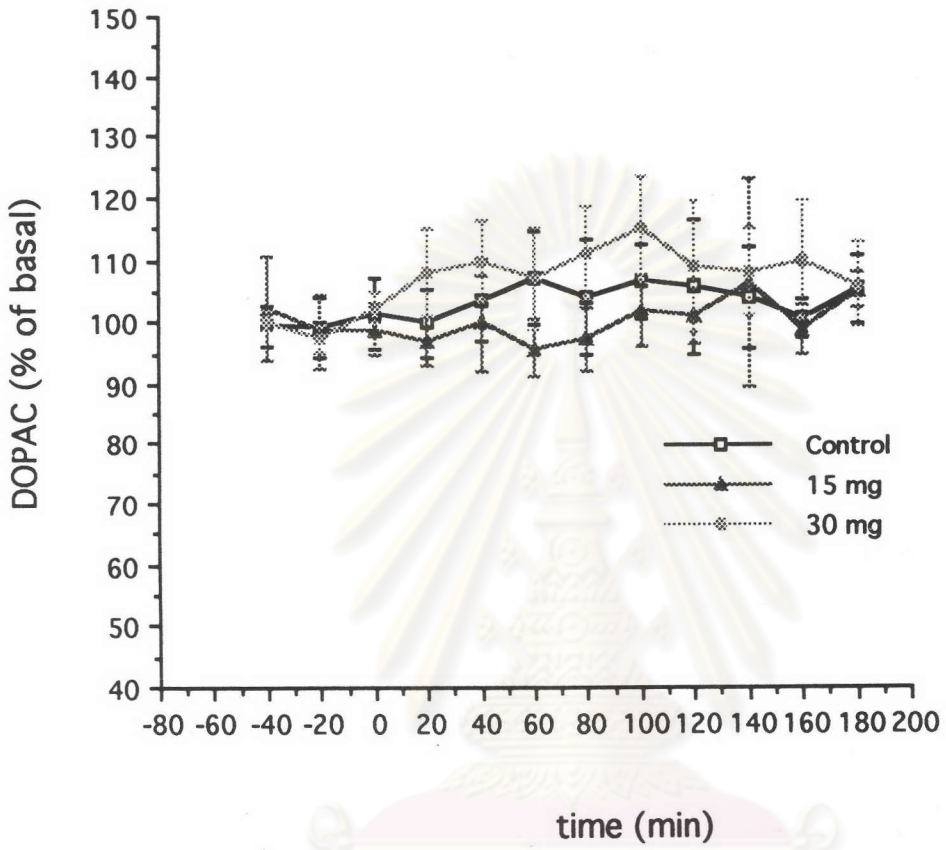


Figure 30. Level of DOPAC in brain dialysate at various time. Mitragynine was injected i.p. at time 0. Control group was injected with 0.5 % CMC in NSS (n=5).

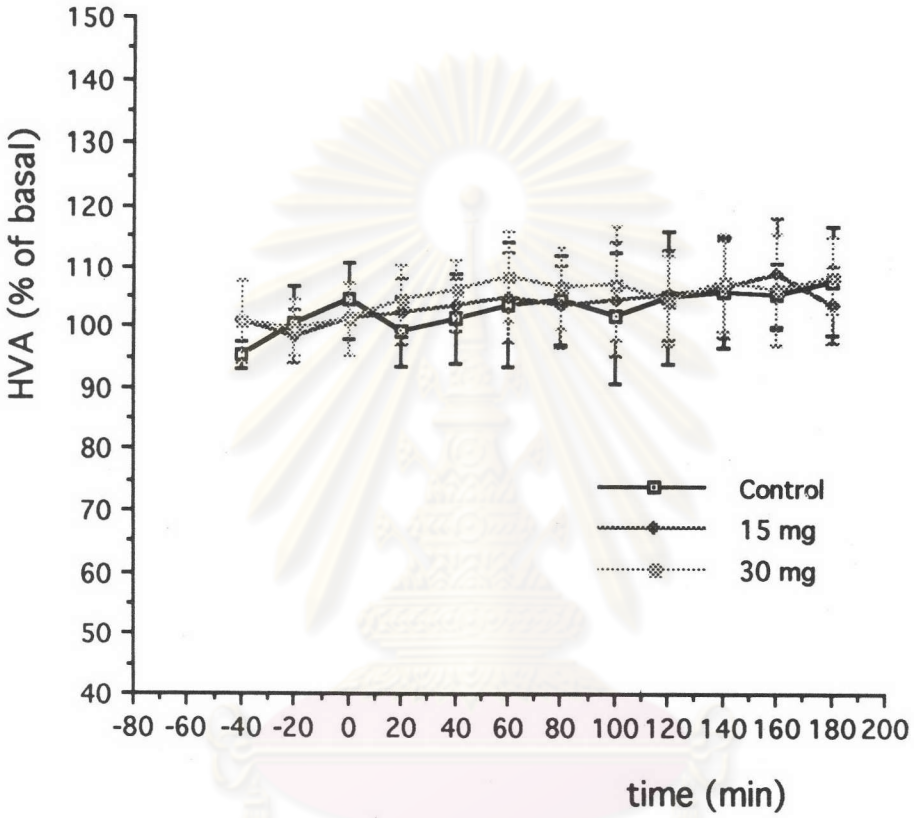


Figure 31. Level of HVA in brain dialysate at various time. Mitragynine was injected IP at time 0. Control group was injected with 0.5% CMC in NSS (n=5).

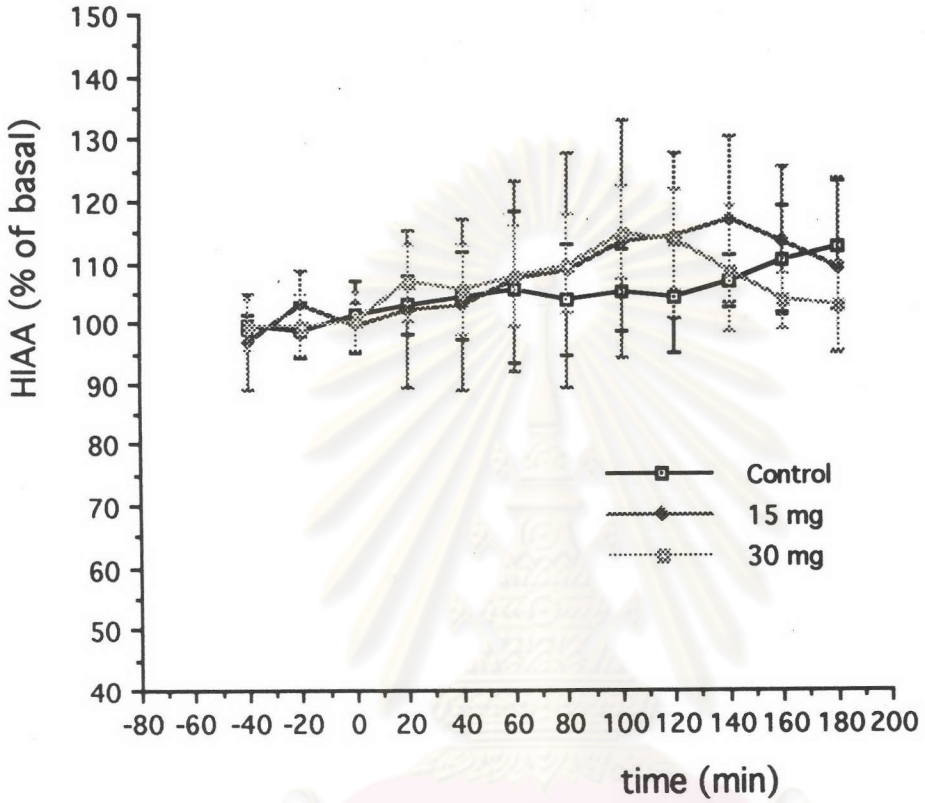


Figure 32. Level of HIAA in brain dialysate at various time. Mitragynine was injected IP at time 0. Control group was injected with 0.5 % CMC in NSS (n=5).

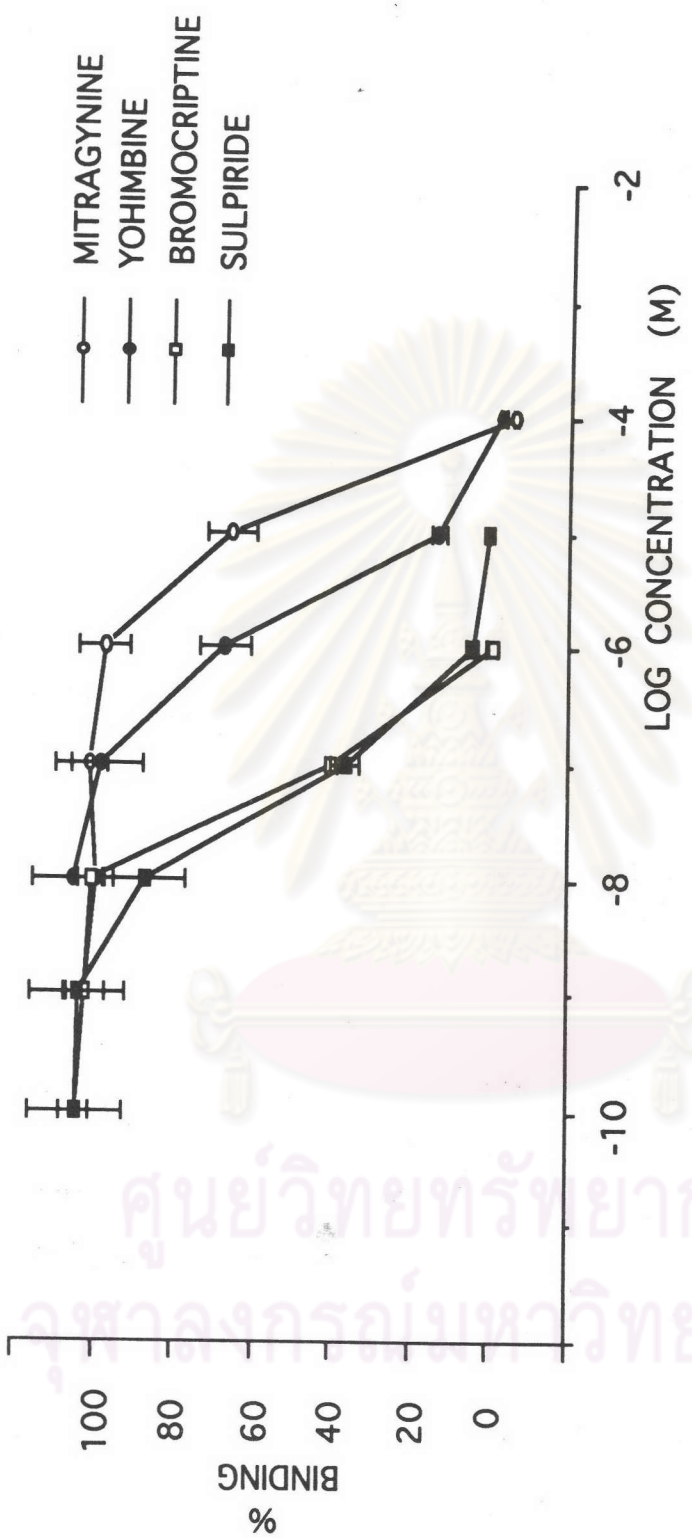


Figure 33. Effect of mitragynine and other substance on the binding of spiperone with dopamine (D2) receptor (n=6).

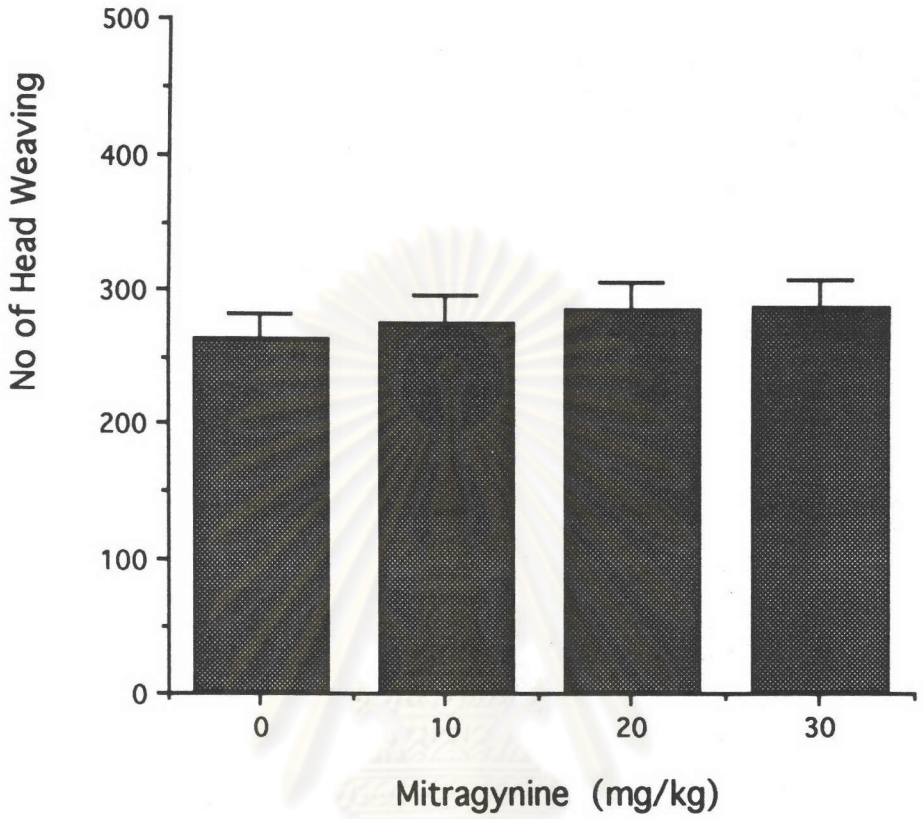


Figure 34. Effect of mitragynine on no. of 5-MeO-DMT induced head weaving in mice. Mitragynine was injected intraperitoneally 30 minutes before 5-MeO-DMT injection. 0.5% CMC in NSS was used as a control (n=10).

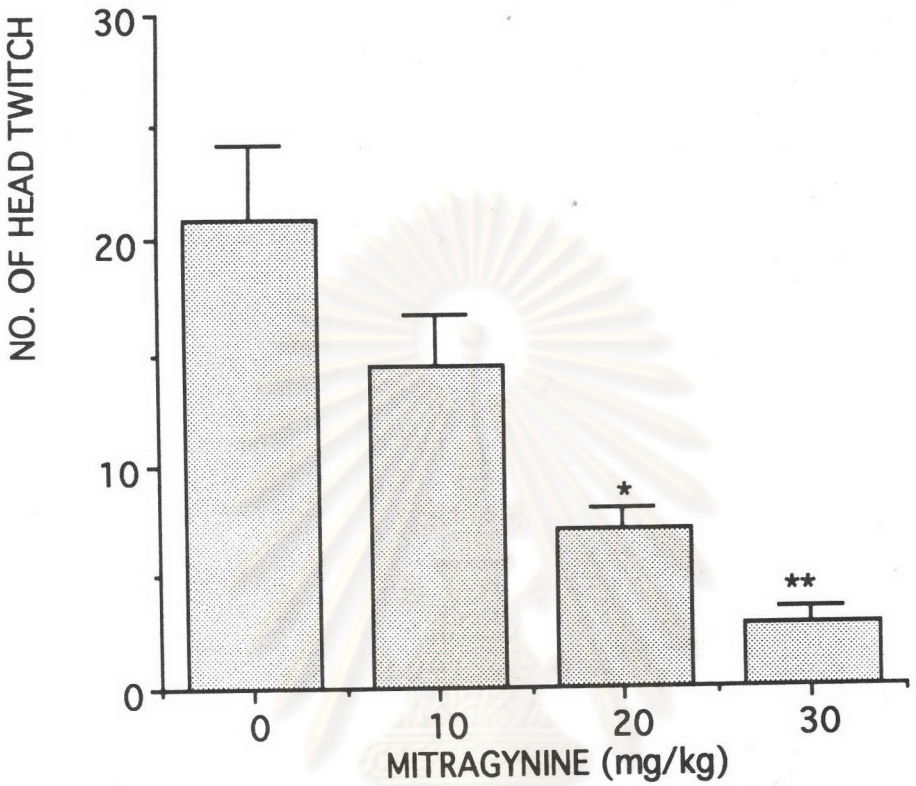


Figure 35. Effect of mitragynine on no. of 5-MeO-DMT induced head twitch in mice. Mitragynine was injected intraperitoneally 30 minutes before 5-MeO-DMT injection. *p<0.05 and **p<0.01 compared with control (n=10)

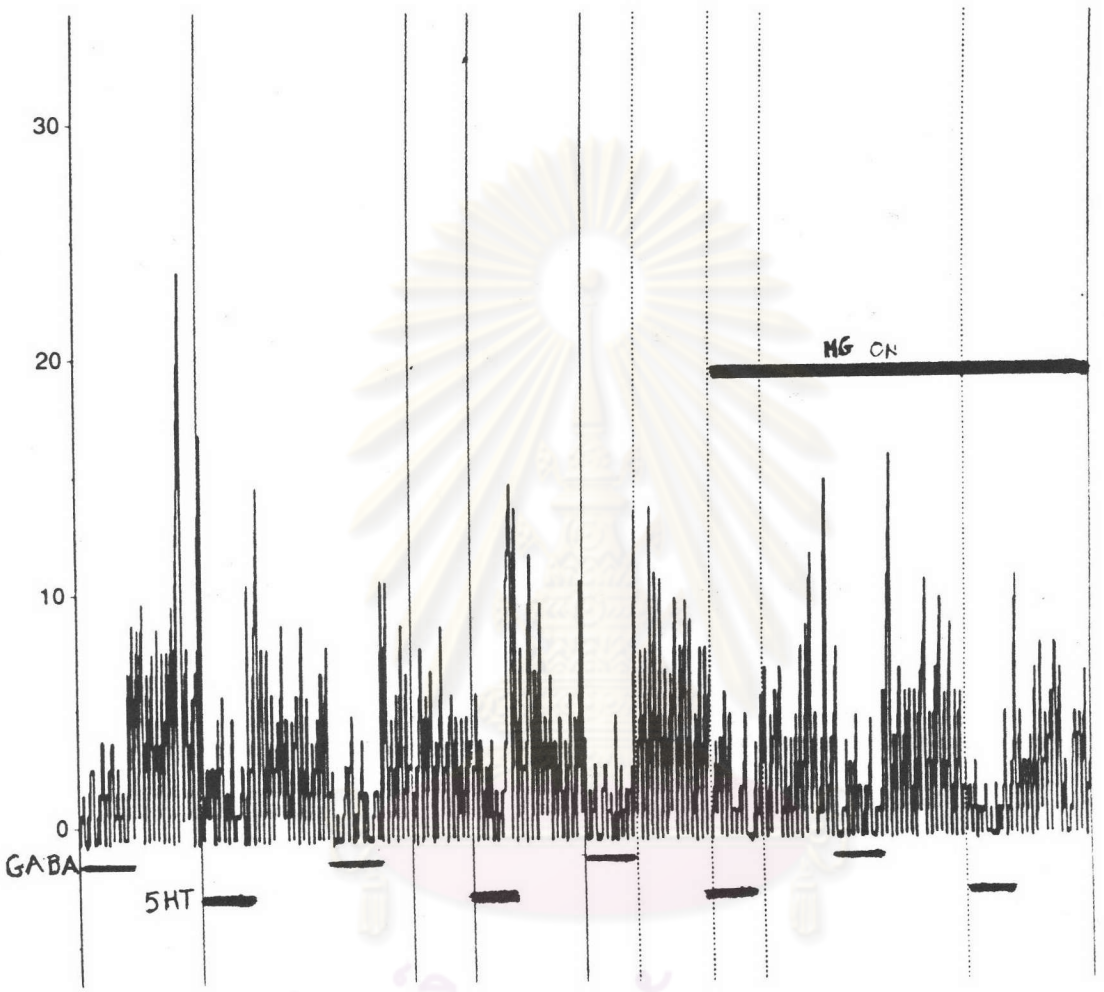


Figure 36. Effect of iontophoretic administration of mitragynine on spontaneous firing rate of Purkinje cell. Horizontal bars indicated administration period of mitragynine (MG), GABA and serotonin (5HT).