การศึกษาปฏิกิริยาไอโอดิเนชันของสารบาราคอลที่สกัดได้จากใบอ่อนของต้นขี้เหล็ก และการทดสอบฤทธิ์ในการลงประจับ

ปริญญานิพนธ์

ของ

กฤษดา เกาะแก้ว

เสนอต่อมหาวิทยาลัยศรีนครินทรวิโรฒ ประสานมิตร เพื่อเป็นส่วนหนึ่งของการศึกษา
ตามหลักสูตรปริญญาวิทยาศาสตร์มหาบัณฑิต สาขาเคมีชีวภาพ
กันยายน 2535
ลิขสิทธิ์เป็นของมหาวิทยาลัยศรีนครินทรวิโรฒ

คณะกรรมการควบคุมและคณะกรรมการสอบได้พิจารณาปริญญานิพนธ์ฉบับนี้แล้ว เห็นสมควรรับเป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตร์มหาบัณฑิต สาขาเคมีชีวภาพ ของมหาวิทยาลัยศรีนครินทรวิโรฒได้

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(รศ.ดร.สุนิพนธ์ ภูมมางกูร)

บัณฑิตวิทยาลัยอนุมัติให้รับปริญญานิพนธ์ฉบับนี้ เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตร์มหาบัณฑิต สาขาเคมีชีวภาพ ของมหาวิทยาลัยศรีนครินทรวิโรฒ

.....คณบดีบัณฑิตวิทยาลัย

(ศ.ดร.สมพร บ้วทอง)

วันที่ 7 เดือน กันยายน พ.ศ. 2535

IODINATION REACTION AND EVALUATION OF SEDATIVE ACTION OF BARAKOL, THE MAIN INGREDIENT EXTRACTED FROM THE YOUNG LEAVES OF *CASSIA SIAMEA* LAMK.

A THESIS

By

KRISANA KAOKEAW

Presented in partial fulfillment of the requirments for the Master of Science degree in Biological Chemistry at Srinakharinwirot University

September 1992

ACKNOWLEDGEMENTS

I wish to express my sincerest gratitude and appreciation to my advisor, Dr. Weerachai Singhaniyom, Head of the Department of Anatomy, Faculty of Medicine, Srinakharinwirot University, for his kindness, valuable supervision, guidance and encouragement throughout the course of this study, and editing of this dissertation.

My deeply gratitude is hereby extended to my co-advisors, Asst.Prof.Dr.Sunan Chainakul, Department of Chemistry, Faculty of Science, Srinakharinwirot University, for her keen interest during the study and kindness in interpretation of the ultraviolet, infrared, nuclear magnetic resonance and mass spectra.

And I am deeply indebted and grateful to Assoc.Prof.Dr. Sunipon Pummangura, Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for his valuable suggestions and discussion in interpretation of the ultraviolet, infrared, nuclear magnetic resonance and mass spectra .

I deeply in gratitude to Asst.Prof.Pongsak Kanluan,Head of the Department of of Physiology, Faculty of Pharmaceutical Sciences, and Mr Chaiyo Chaichantipyuth, Head of The Department of Pharmaconocy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for their kindness , guidance and encouragement in laboratory work.

My deeply gratitude is extended to Dr. Saisanom Thammapituk, Department of Chemistry , Faculty of Science, Srinakharinwirot University, for valuable supervision, guidance and encouragement during the course of study in this University .

Finally, I wish to express my appreciation and gratful to my father, my mother, for their love and understanding encourage me in every ways, I ar so sincere thanks.

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ABBREVIATIONS

dec.	=	decompose
^{1}H-NMR	=	Proton nuclear magnetic resonance
ip.	=	intraperitoneal
LD_{50}	=	Lethal Dose-50
MH_Z	=	mega hertz
m.p.	=	melting point
ppm	=	part per million
S.E.M.	=	Standard error of mean

CHAPTER I

INTRODUCTION

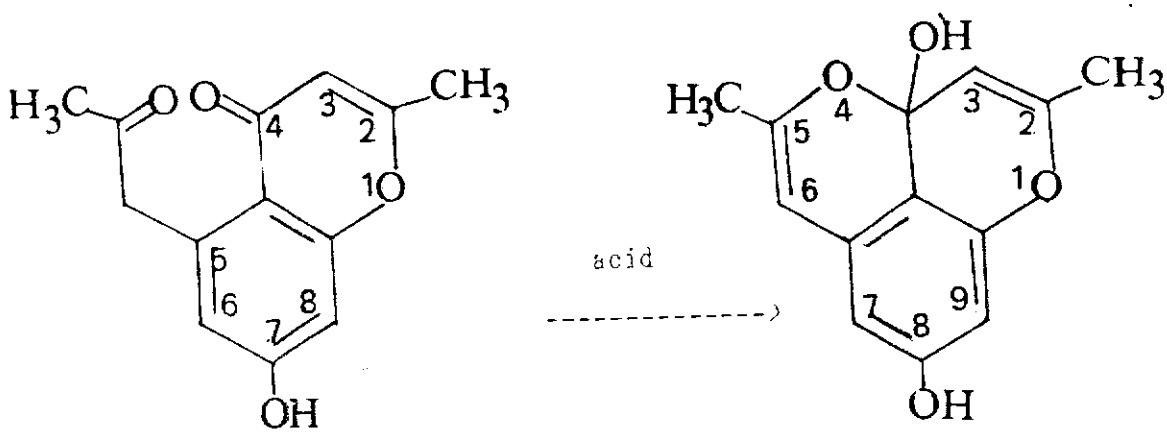
Cassia siamea Lamk. is in the genus Cassia, Family Caesalpiniaceae and found growing throughout the tropical countries.In Thailand, it has various local names depending the growing areas such as in the central part it is called "Khi-lek-loung" ,"Khi-lek-ban" or "Khi-lek" in the south it is called "Ya-ha".

This plant is a low tree with 8-9 meters in height. Its leave has a pinnate type with 4-12 pairs of leaflets and 3-4 cm. The leaflets is oblong shape and 3-4 cm. long. The apex is often rounded or emerginate. The flowers are yellow and have long spike. Its pod has in flat shape and longitudinally waved (Figure 1; Larsen. 1977. and Pongboonrod. 1981.).

Generally, the young leaves and flowers of *Cassia siamea* have been widely used as vegetable in Thai cooking. Moreover, its parts have also been used as ingredients of drugs in traditional medicine. The root and bark have been widely used as anthelmintic and antipyretic drugs. While the young leaves and flowers have been used for treatment of insomia (Kittikajorn. 1983., Pongboorod. 1950. and Arunlakshana. 1949.). The depressive effect on the central nervous system, particular cerebrum and spinal cord have been reported in animal after treatment with crude ethanol extracted of the leaves (Arunlakshana. 1949.) The respiration was apparently unaffected by this substance in rat even after treatment with very large dose. The crude ethanol extract slightly increased stomach smooth muscle and heart muscle contraction in the isolated organ study. Likewise, the crude ethanol extract slightly increased arterial blood pressure after intravenous injection (Arunlakshana. 1949.).

Figure 1 Photograph show leaves and flowers of *Cassia siamea* Lamk.

Chromone is the major chemical ingredient isolated from the leaves of *Cassia siamea* and its chemical structure has been evaluated by Arora (Figure 2 ; Arora. 1971.). It was found later that this chromone reacts with acid (Figure 2 ; Wagner. 1978.) and transforms to 3a, 4-dihydro-3a, 8-dihydroxy-2, 5-dimethyl-1, 4-dioxaphenalene which is called barakol (Hassanali. 1969.). The processes of isolation of the barakol was later improved by acid extraction (Chaichantypyth. 1979.) which gave better yield (0.1 %).



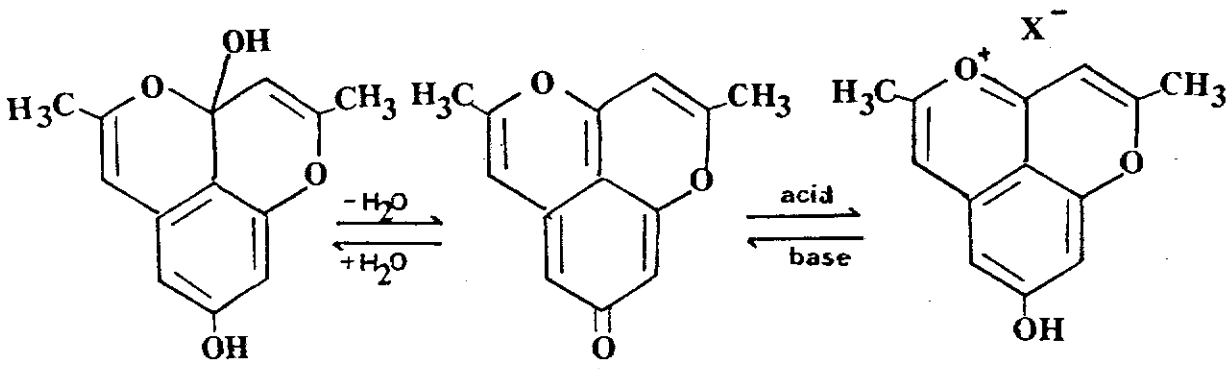
5-acetonyl-7-hydroxy-2-methylchromone

Barakol

Figure 2 Acid treatment of 5-acetonyl-7-hydroxyl-2-methylchromone into barakol.(Arora. 1971.)

Barakol ($C_{13}H_{12}O_4$) is a pale lemon-yellow needle crystal and has melting point at 165 °C. This substance is readily soluble in methanol, ethanol and acetone moderately soluble in chloroform and dichloromethane and readily soluble in benzene, carbontetrachloride, ethyl acetate and water (Hassanali. 1969.). The barakol is usually

extremly unstable in normal condition by lossing water molecule and become the dark green amorphous compound anhydrobarakol. However, this substance can be easily reconversed to barakol by dissolving in aqueous methanol (Bycroft. 1970.). However, it stability can be improved by addition of concentrated hydrochloric or hydrobromic acid to a methanolic solution of barakol, giving anhydronium salt. Chemical structures of barakol, anhydrobarakol and anhydronium salt have been evaluated by spectroscopic studies (Figure 3 ; Bycroft. 1970.).



Barakol Anhydrobarakol Anhydronium salt

Figure 3 Conversion reaction among barakol, anhydrobarakol and anhydronium salt.

Pharmacological properties of the salt form of barakol such as anhydrobarakol hydrochloride have been later evaluated and found that the barakol can decrease locomotor activity in mice (Jantarayota. 1989.). Then, it was suggested as central nervous system depressant due to the inability to suppress central nervous system stimulation drugs such as picrotoxin, bicullin, and strichnine. The toxic effect of

barakol is minimum since the LD_{50} (324.09 mg/kg) (Jantarayota. 1989.). And apart from sedative effect, this compound showed very low antimicrobial activity (Gritsanapan. 1989.). Several literatures related with barakol suggested that barakol may be a potentially good candidate for natural sedative, analgesic drug. The advantages of the barakol is less toxic and cheap due to the availibility of large supply. However, the employment of this substance as the drug requires more detailed studies and clinical trials to ensure its properties pass all basic regulations. One of important informations concerning which receptors in the central nervous system reacts with the barakol and induces the sedative effect is primarily needed. This solution can be easily by the receptor autoradiographic technique which needs the radiotracer as a marker. Thus, it is important to prepare the radiotracer for this study by substituting radioisotope (^{3}H or ^{125}I) into the barakol molecule.

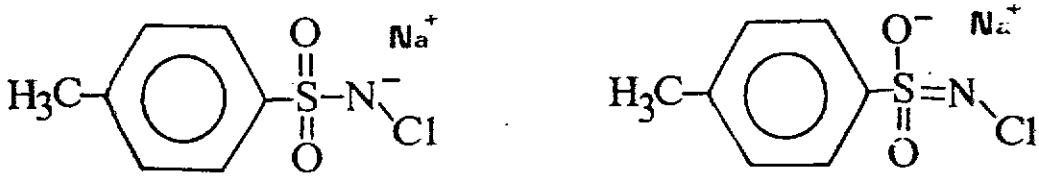
The radionuclide ^{125}I or ^{131}I is widely used for the preparation of tracer for sensitive receptor autoradiographic technique and other procedure for detection, localization and quantitation of substances in biological samples. In the preparation of tracer, in the case of preparation protein labelled compound for radioimmunoassay technique, radioiodine can be introduced into protein either directly using, for example the soluble oxidizing agent chloramine T , indirectly by conjugating to a ready-labelled compound such as ^{125}I- Bolton and Hunter reagent. The biological properties of the prepared tracer must be investigated whether it still retain the properties of unlabelled material (Bolton. 1989.).

The chloramine T method is the most widely used for the radioiodination of small mass of protein and other substances to high specific radioactivities for use as tracer in receptor

autoradiographic technique and other procedures. The chloramine T reaction is technically simple and rapid to perform and is thus the method of choice in the first instance when setting out to iodinate a protien for the first time (Sherman, Harwig and Hayne. 1974.). Chloramine T is the sodium salt of the N-monochloro derivative of p-toluene sulphonamide. The structure of chloramine T is most commonly depepicted as I and occasionally as II (Figure 4 ; Malcolm and Johnson. 1978.). Chloramine T is stable in aqueous solution, it is strong electrolytes and is strong oxidants in both acidic and alkaline media. In aqueous solution it thought slowly breake down to toluene sulphonamide and hypochlorus acid is form (Figure 5 ; Krisana, Verma and Gupta. 1982.).

slow

chloramine T ----------> HOCl + toluenesulphonamide



I II

Figure 4 Chemical structure of chloramine T

A hypochlorus acid is consequently a mide oxidizing agent. In the presence of chloramine T under midly alkaline condition (pH 7.5) NaI is oxidized forming cationic iodine, I^+. And at this the ortho position in the aromatic ring of tyrosine of peptides and protein is activated for electrophilic attack, owing to the electron-donating effect of the neighboring hydroxyl group. (Due. 1964.) The monoiodo and diiodo derivative of tyrosine has a lower pK, about 8.5 and is thus more highly ionized at the pH of the iodination reaction, although it is less reactive after mono- and di- substitution. The iodine atoms can substitute at the ortho positions to the hydroxyl group in the phenolic ring of tyrosine. (Figure 5 ; Seon. 1970.)

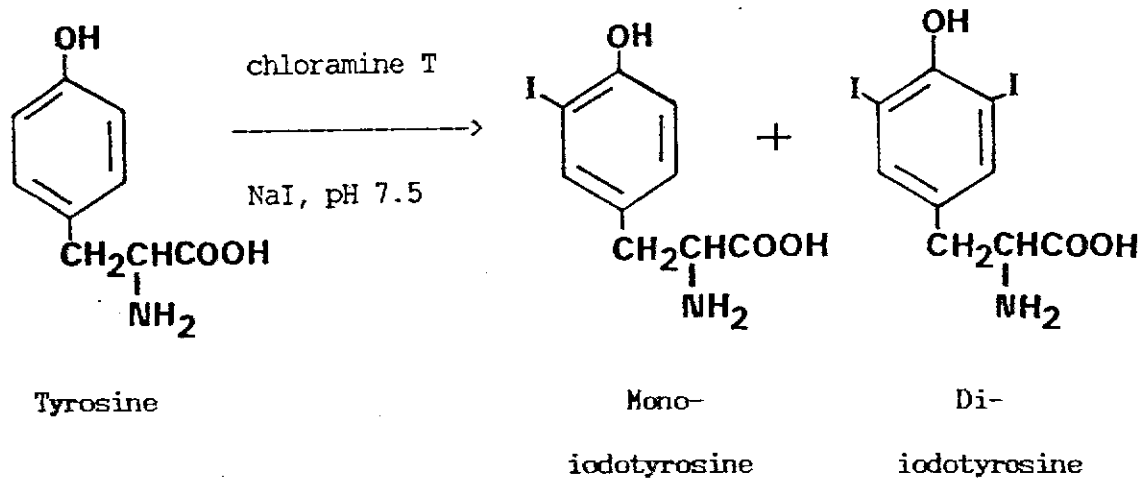
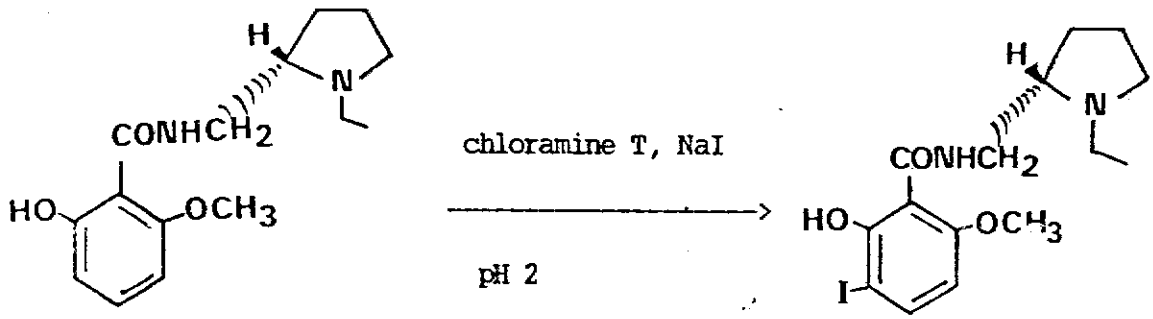


Figure 5 Iodination reaction of tyrosine by chloramine T method.

Kung(1988), prepared the IBZM ; a potentail CNS D-2 dopamine recepor by chloramine T method at pH 2. (Figure 7)



BZM IBZM

Figure 6. Iodination reaction of benzamide by chloramine T method.

The aim of this study is to investigate iodination reaction of the salt form of barakol such as anhydrobarakol hydrochloride using chloramine T method. Whether, there is possibility to introduce iodine atom into the ring structure of anhydrobarakol hydrochloride. If possible, whether the reaction product still retain sedative property. Information obtained from this study is primarily required for the preparation radioiodinated compound from the anhydrobarakol hydrochloride and this will be used as radiotracer in the receptor autoradiographic technique.

CHAPTER II

EXPERIMENTAL

General Procedures

Melting points were determined on a Buchi melting point apparatus.

Infrared spectra were registered in KBr pallets with Jasco IR-700 spectrophotometer.

Ultraviolet spectra were determined in ethanol on a Shimadzu UV-160 spectrophotometer.

^{1}H-NMR spectra were recorded on a Cryomagnetic For Spectroscopy BZH 200/52 (200 MH_Z) spectrometer, using tetramethylsilane (TMS) as the internal standard.

Mass spectra were measured with JEOL JMS-DX 300 mass spectrometer.

Thin layer chromatography was performed on 250 x 750 x 0.25 mm layers of silica gel G60 F254 (E.Merck),activated at 100°C for an hour, and protectively stored in a desicator.

Column chromatography was carried out on silica gel(E.Merck, 70-230 mesh). All solvents were distilled before chromatography.

Removal of solvents was effected using a Buchi rotary evaporator connected to a water pump.

This part of study consists of three parts namely, preparation of barakol, iodination reaction of anhydrobarakol hydrochloride and verification of animal locomotion activity.

1. Preparation of barakol.

1.1 Plant Materials.

Fresh young leaves of Khi-lek (*Cassia siamea* Lamk.) were obtained from Nakhonchaisri central market in Nakhonprathom province, Thailand in December 1991. The plant materials were identified by comparison with the herbarium specimens in the Botany section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand.

1.2 Solvents and chemicals.

- Sulphuric acid,concentrate.
- Acetic acid,glacial.
- Ammonium hydroxide,strong solution.
- Sodium hydrogen carbonate.
- Ethanol
- Methanol
- Chloroform

1.3 Procedure

Two kilogrames of fresh young leaves of *Cassia siamea* Lamk. were blended into small pieces and mixed with 0.5 % H_2SO_4. (8 litre) The mixture was then heated until boiling and left for 30 minutes, prior to filtration. The filtrate was alkalinized with sodium hydrogen carbonate and extracted by chloroform (15 litre). The chloroform extract was concentrated by evaporated under reduced pressure to 500 ml, and mixed with the equal volume of distilled water. The mixture was shaken strongly by hand to allow precipitation of the yellow lemon needle crystals then left the solution in cooling bath for an hour for complete precipitation. The yield was approximately 8 g. The crystal was identical in Rf value on TLC when using silica gel coated TLC plate,

the mixture of chloroform and methanol (9:1) and a mixture of chloroform and acetone (6:4) were employed as the solvent system.The crystals were purified by several recrystallization in 5% acetic acid and neutralized with strong ammonium hydroxide solution. The final yield of presumed barakol was approximately 6 g which was designated as substance B (Figure 8).

The substance B was dissolved in minimum amount of methanol and the concentrated hydrochloric acid was later added. The presumed anhydrobarakol hydrochloride was slowly crystalized in yellow needle form. The crystal was recrystallized several times by aqueous ethanol. This product was designated as substance B.HCl.

The chemical structure of substance B and substance B.HCl were identified by pattern of spectroscopy spectra and compared with those of previous works.(Bycroft. 1970.)

2. Iodination reaction of anhydrobarakol hydrochloride.

The substance B.HCl was reacted with sodium iodide , using chloramine T as the oxidizing agent.

2.1 Solvents and chemicals.

- Chloramine T
- Sodium iodide
- Hydrochloric acid solution at pH 2
- Sodium acetate -acetic acid solution at pH 3, 4, 5 and 5.89
- Phosphate buffer solution at pH 7.5
- Ammonium hydroxide ,strong solution
- Chloroform
- Ethyl acetate
- Ethanol
- Methanol

2.2 Procedure

Twenty millilites of chloramine T solution (mg/ml) was added to a mixture containing 25 ml of anhydrobarakol hydrochloride solution (2 mg/ml), 10 ml of sodium iodide solution (mg/ml) and 100 ml of HCl solution (pH 2). The mixture was shaken at room temperature (25- 27 °C) until the brown color of iodine was disappeared. Then, the brown precipitation was gradually precipitated within 1 to 2 minutes, the reaction was left for 30 minutes for complete precipitation. The product from the reaction was identical in Rf values on TLC when using silica gel coated TLC plate, a mixture of chloroform and methanol (9:1), a mixture of chloroform and ethyl acetate (6:4) and a mixture of chloroform and acetone (5:5) were employed as the solvent system. The precipitation was later filtered by filter paper NO.1 and dissolved in chloroform prior to the seperation by column chromatography using silica gel as the adsorbent. A mixture of chloroform and ethyl acetate (6:4) was employed as the solvent system. Fraction containing product were collected and evaporated under reduced pressure until the yellow-brown crystalline was precipitated ; it was designated as substance A1. The supernatant was neutralized with ammonium hydroxide solution and later extracted by chloroform which subsequently was concentrated by evaporated under reduced pressure until dried. The product from the reaction was identical in Rf values on TLC when using silica gel coated TLC plate, a mixture of chloroform and methanol (9:1), a mixture of chloroform and ethyl acetate (6:4) and a mixture of chloroform and acetone (5:5) were employed as the solvent system. The product was dissoved in chloroform prior to seperated by column chromatographic technique. Fraction containing the product were collected and evaporated under reduced pressure until

the yellow crystalline was precipitated ; it was designated as substance A2. The A1 and A2 were further purified by recrystallization with absolute ethanol.

Iodination reaction was also performed at pH 3, 4, 5, 5.89 and 7.5. Processes of seperation and purification of the products were the same as those in 2.2.

3. Verification of animal locomotion activity of anhydrobarakol hydrochloride and substance A1.

3.1 Animals : Male swiss mices at 4-8 weeks of age and weight ranging between 30-40 gms were used in experiments. Each experiment employed 6 mices.

3.2 Instrument and chemical : Syring (1 ml) with injection needle(26G) and normal saline solution.

3.3 Apparatus : Animal Locomotor Activity Monitoring System, consist of test chamber surrounded by sensors. The test chamber is square in shape (each side = 40 cm.) and 20 cm in hight. The sensors consists of two single row of eight photoelectric cells and these cells will receive the light signals from the infrared cells (8 cells lied in a single row)in the opposite side of the chamber(Figure 7). The movement of animals in the chamber will be detected by the infrared light and was recorded by the photoelectric cells at 5 minutes interval.

3.4 Procedure :

The experiments were carried on as follw ;

Day 1 : Mice was placed into the test chamber for 30 minutes, to get acquainted with the new environmental condition. Then the mice was injected with .0.1 ml normal saline solution and left for 120 minutes for normal locomotion activity recording. The number of activity counts were plotted against time at 15 minutes intervals.

Day 2 : The mice was injected with 0.1 ml of substance B.HCl(ip., dose 20, 40, 60, 80 and 100 mg/kg)or substance A1 (dose 60 mg/kg) instead of 0.1 ml normal saline and left for 120 minutes. The number of activity counts were plotted against time at 15 minutes interval.

All experiments were performed during 7.30-10.00 AM. of each day.

3.5 Statistics :

Comparisons of data between the control and the treated mice were made calculated using the Willcoxon match pairs test. Comparisons between anhydrobarakol hydrochloride-treated and substance A1-treated animals were calculated using Mann whitney U´test. Both of methods were analyzed at a P value of 0.05 (two - side) and were considered as significant difference.

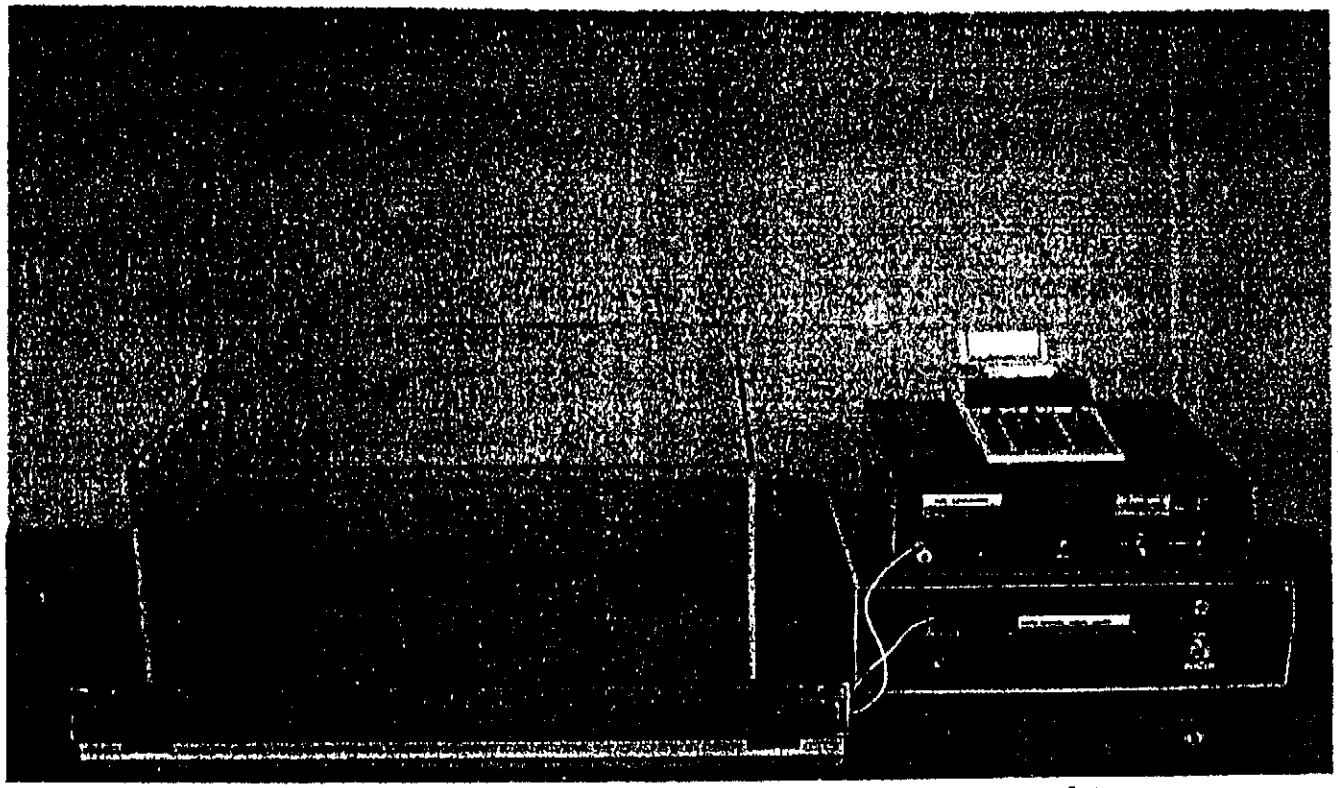


Figure 7 Photograph show Animal Locomotor Activity Monitoring System.

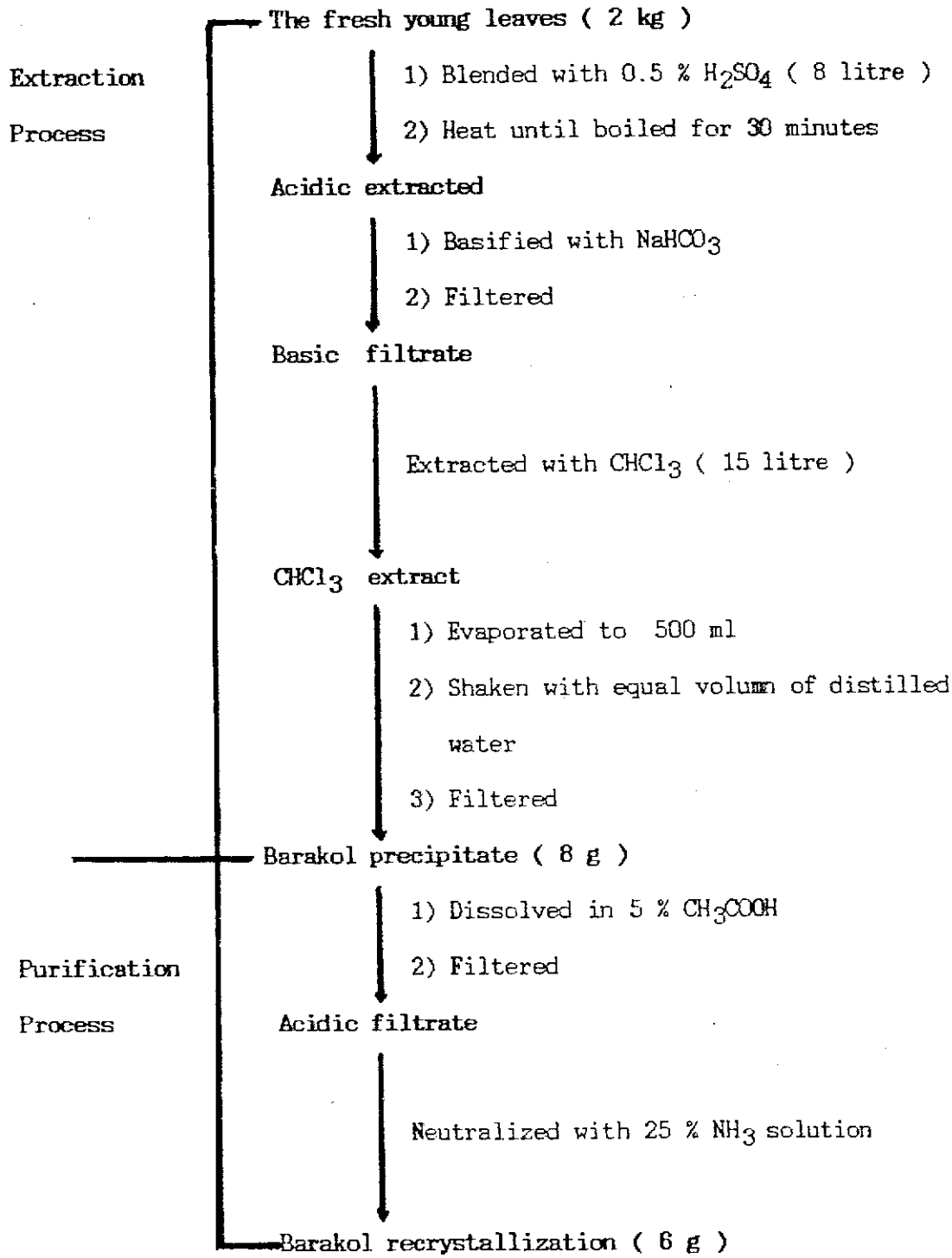


Figure 8 The Barakol extraction and purification procedure of *Cassia siamea* Lamk.

CHAPTER III

RESULS

1.Preparation of Barakol

The extraction of the shredded fresh young leaves of the *Cassia siamea* Lamk. with sulphuric acid (0.5 %) followed by alkalinization with sodium hydrogen carbonate produced the pale lemon -yellow crystals.These reactions gave 0.3 percent yield of the product(substance B), presumably, barakol. Then, the product was further reacted with concentrated hydrochloric acid, giving 70 percent of anhydronium salt (substance B.HCl), presumably, anhydrobarakol hydrochloride which is the yellow needle crystals. The Rf value showed at 0.3 when using silica gel G60 F254 as the absorbent and the mixture of chloroform and methanol (9:1) as solvent system, however Rf value was showed at 0.5 when using the mixture of chloroform and acetone (6:4) as the solvent system. The physical and spectroscopic characteristics of the products were evaluated ; mp. 164-165°C (dec.) ; UV λ_{max} (EtOH) nm(log ε): 240(4.8) and 390(4.52) (Figure 9) ; IR ν_{max} (KBr) 3450, 1670,1560and1470 cm^{-1} (Figure 10) ; ^{1}H-NMR($CDCl_3$+ DMSO-d_6) : δ 6.01 (1H,d,H-9), 6.00 (1H,d,H-7), 5.99(1H,s,H-6), 5.81(1H,s,H-3), 2.07,1.94 (6H, 2xs, 2xMe) (Figure 11) ; MS, *m/z* (rel.): M^+, 232 (>1), 214 (98), 186 (69), 158 (26),143 (8), 115 (23), 93 (20), 89 (16), 51 (19), and 43 (100)(Figure 12).

The physical and spectroscopic characteristics of substance B.HCl were also evaluated ; mp. 208 °C (dec.) ; UV λ_{max}(EtOH) nm(log ε) : 240(4.96) and 384(4.54) (Figure 13) ; IR ν_{max} (KBr) 3500, 2740, 1700, 1600, and 1442cm^{-1} (Figure 14) ; ^{1}H-NMR (D_2O) : δ6.60(2H,d,H-9,H-7), 6.60 (1H,s,H-6), 6.40 (1H,s,H-3), δ 2.47, 2.33 (6H, 2 x s, 2 x Me)(Figure 15).

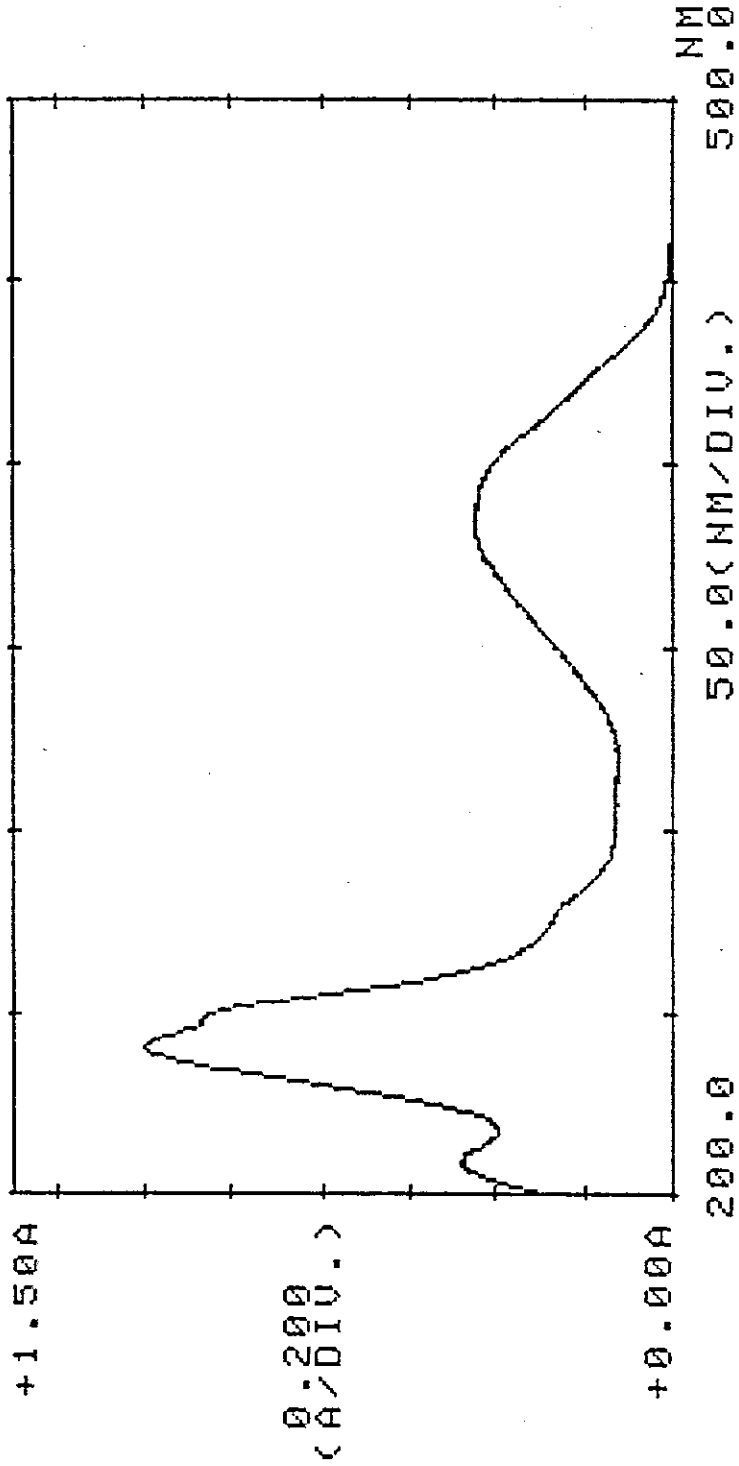


Figure 9 UV absorption spectrum of substance B, in ethanol.

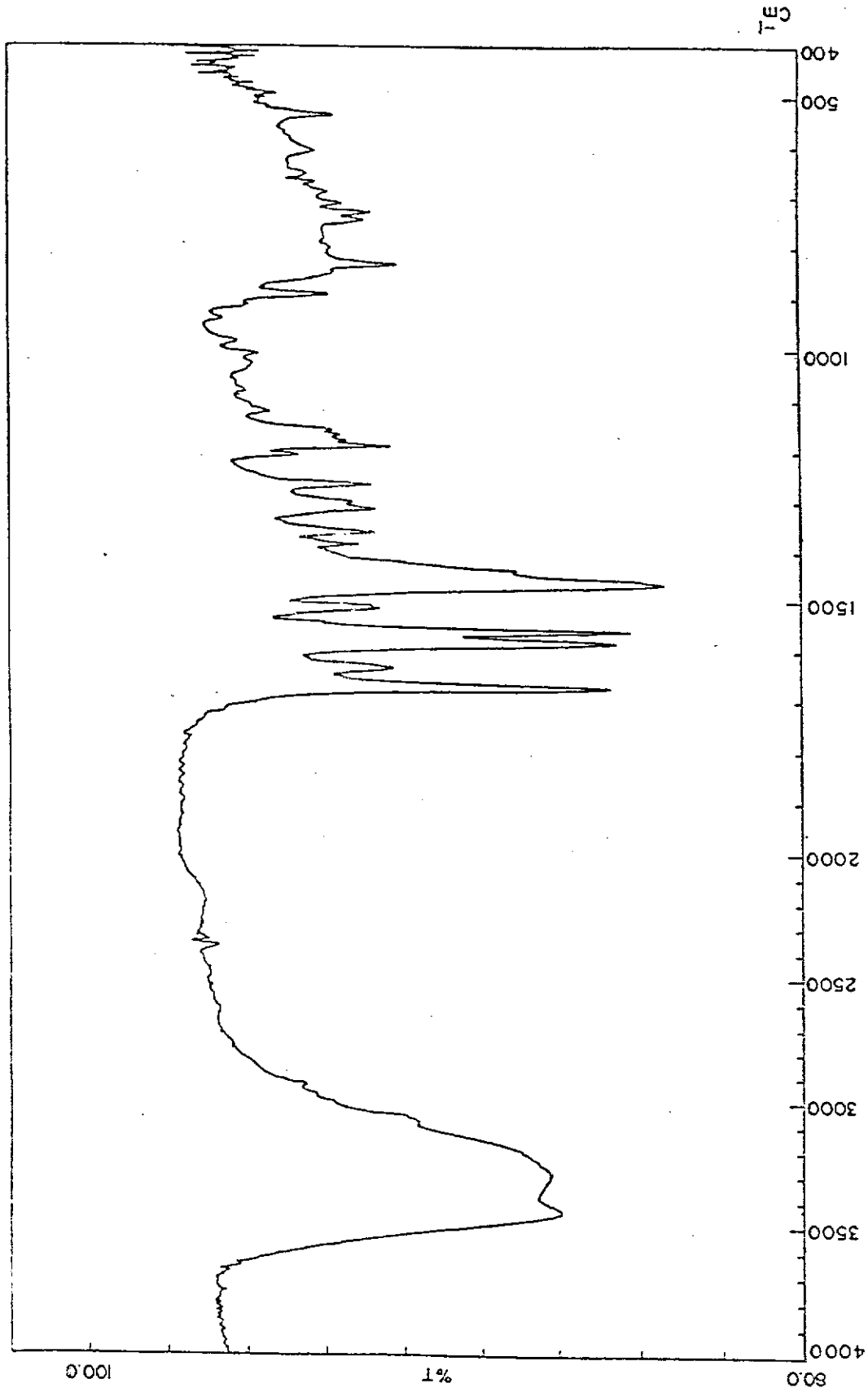


Figure 10 IR absorption spectrum of substance B, in KBr.

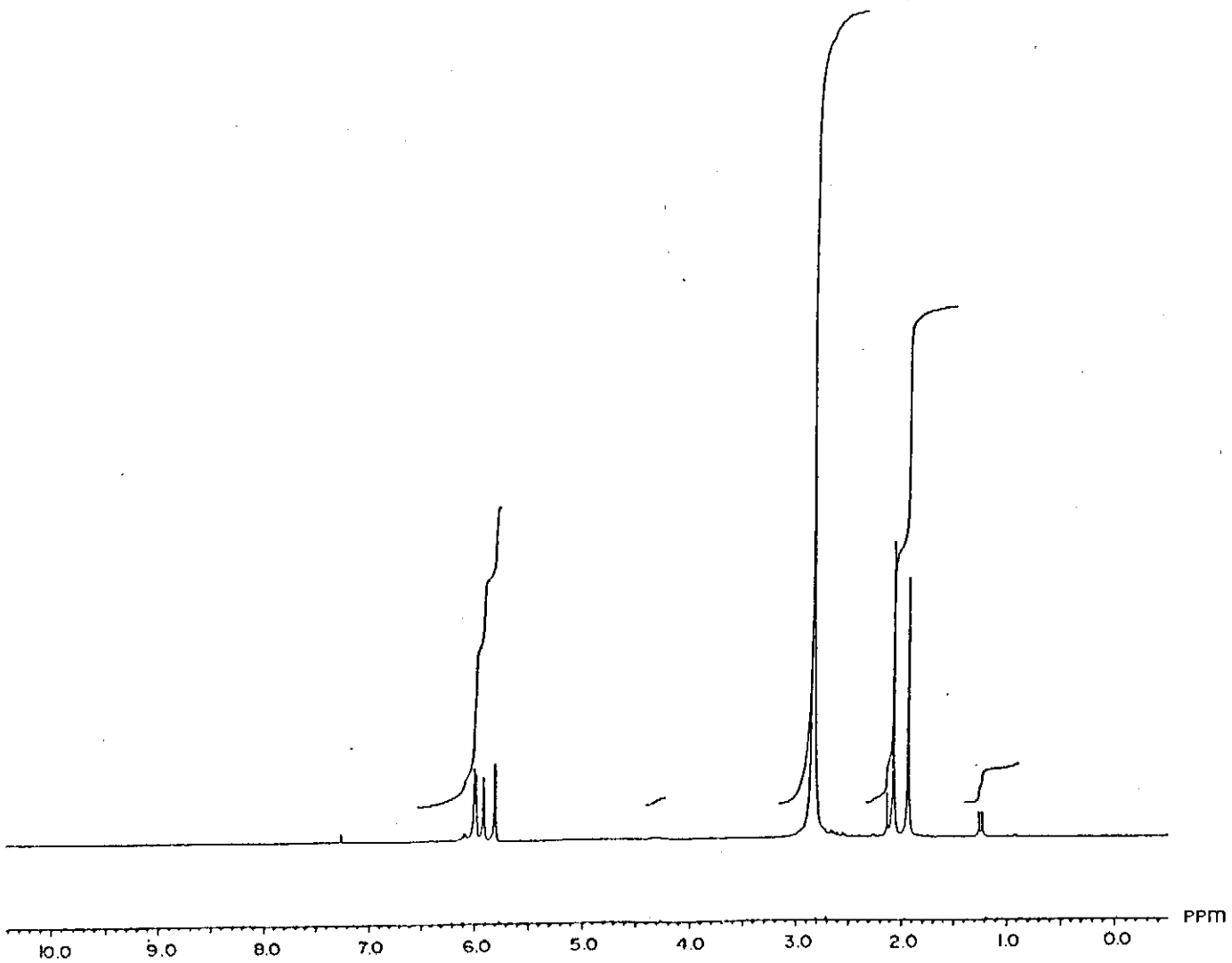


Figure 11 ^{1}H NMR spectrum of substance B, in $CDCl_3$+ DMSO-d_6 (at 200 MH_Z).

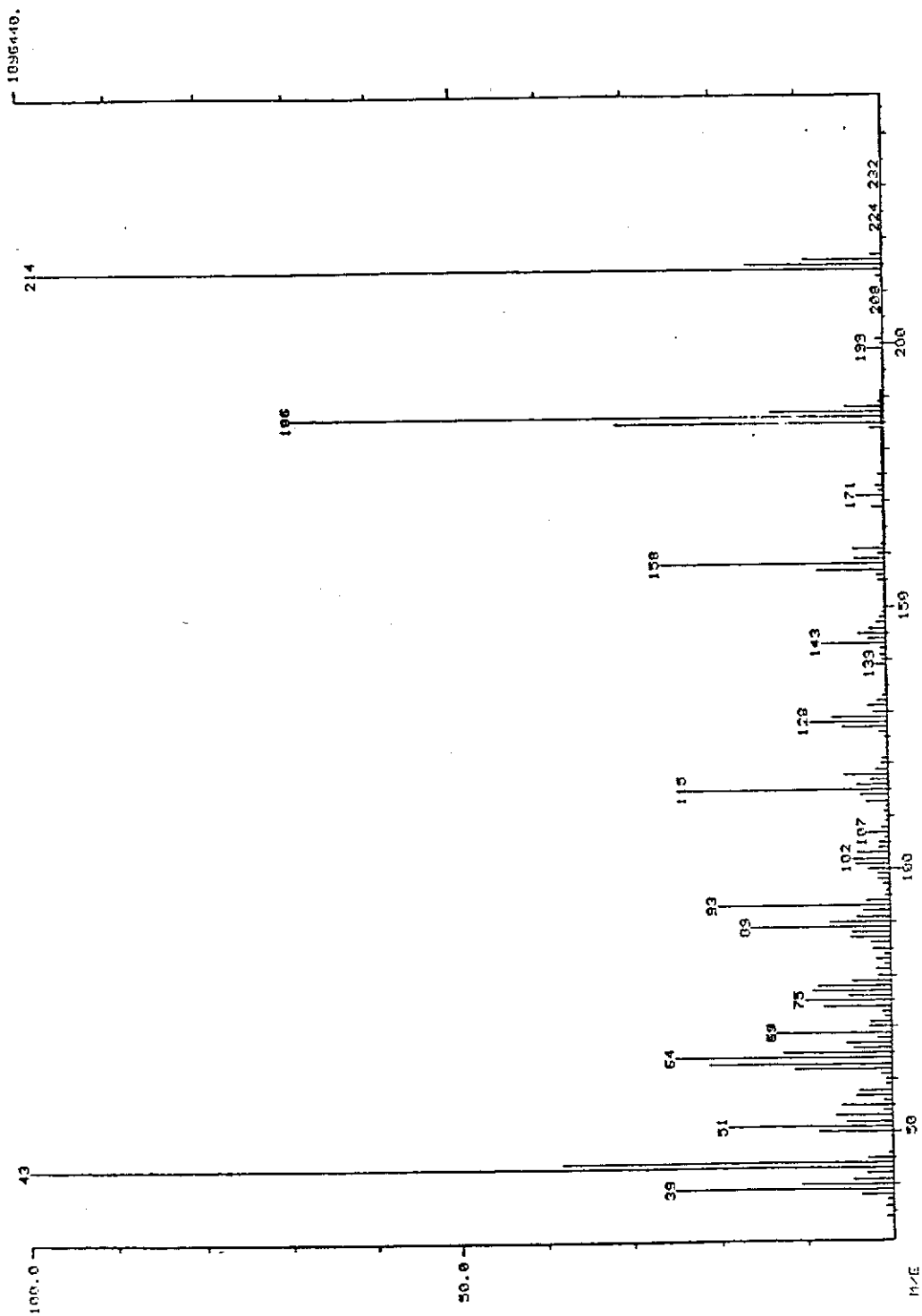


Figure 12 Mass spectra of substance B.

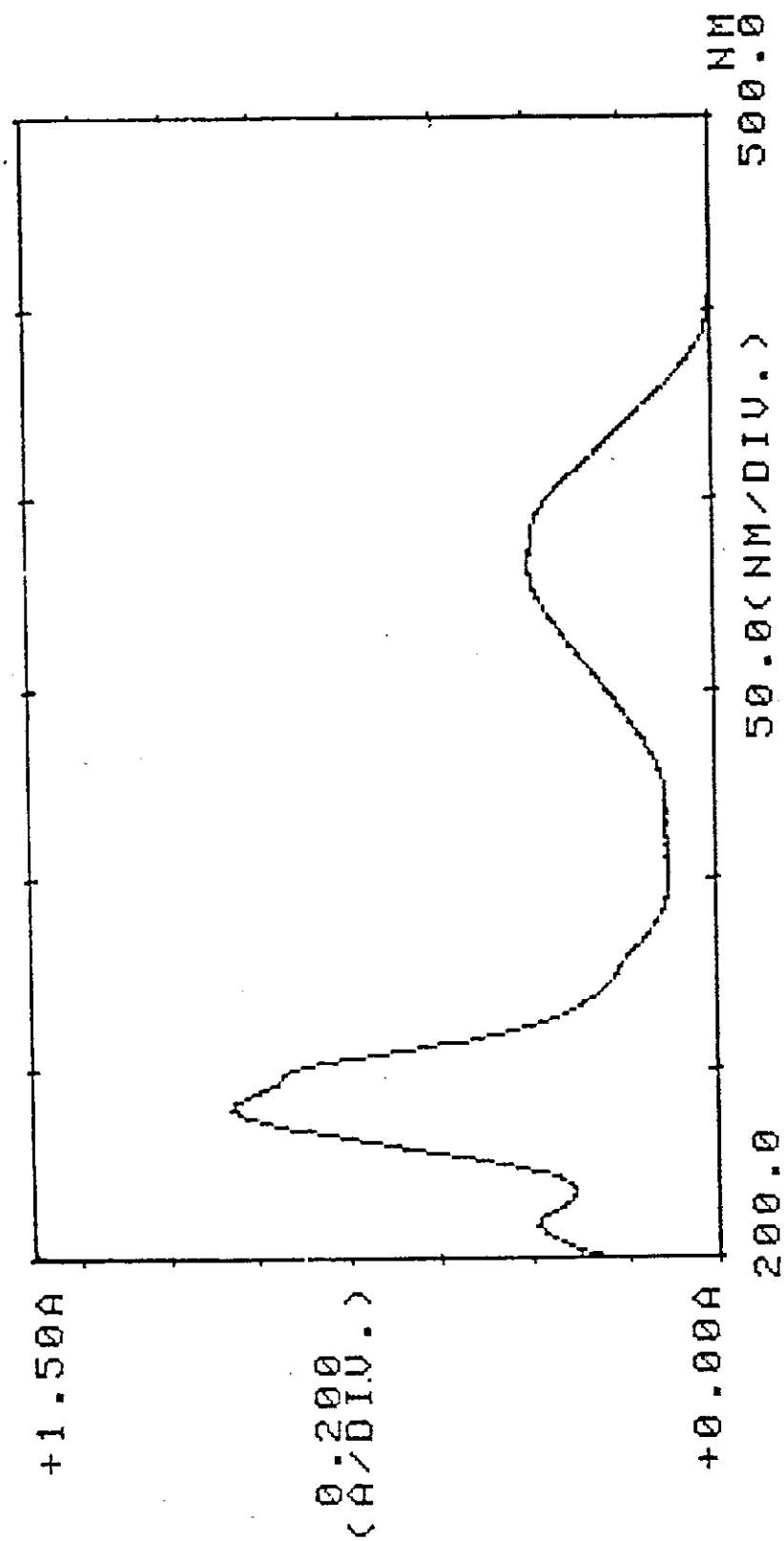


Figure 13 UV absorption spectrum of substance B.HCl, in ethanol.

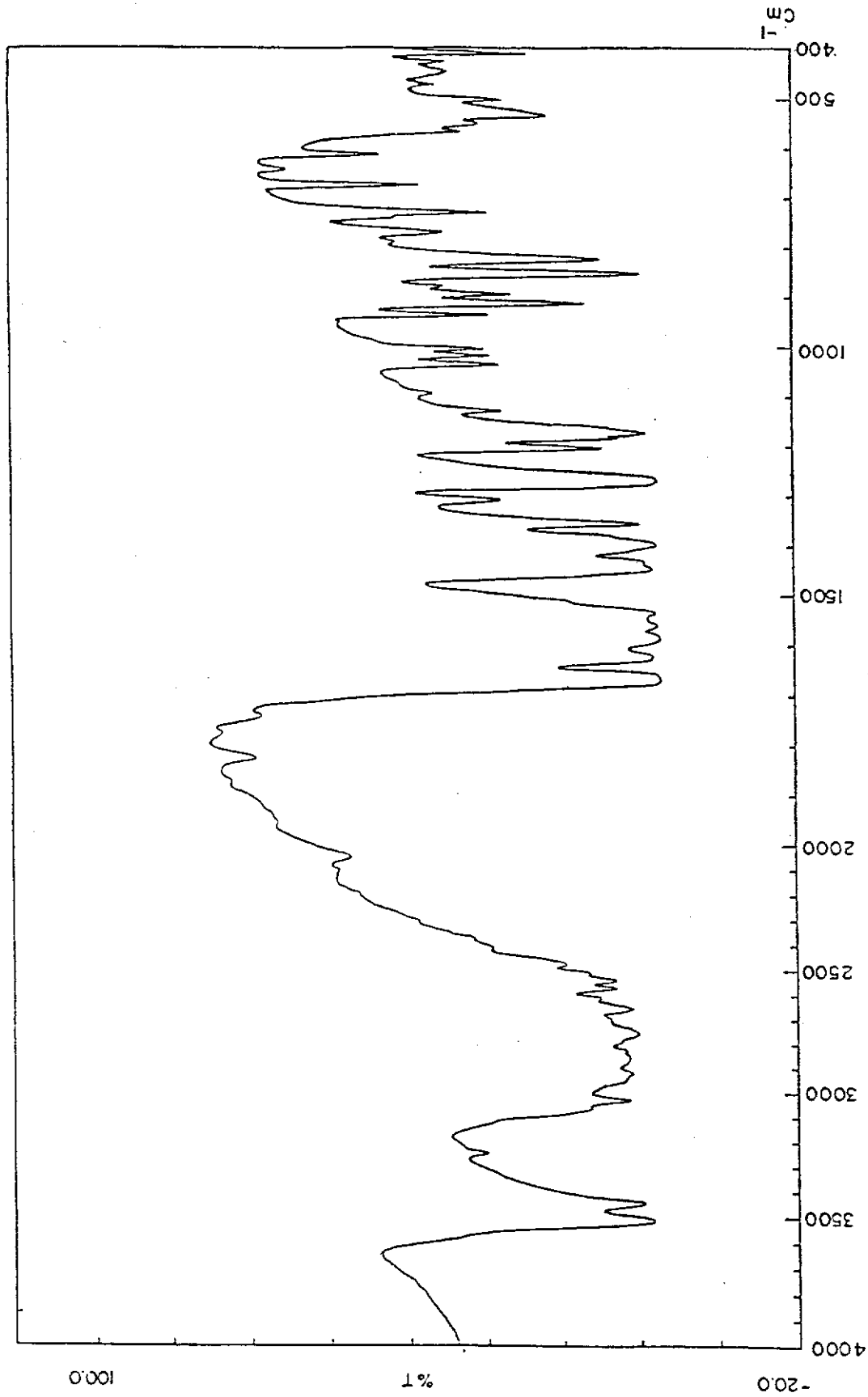


Figure 14 IR absorption spectrum of substance B.HCl, in KBr.

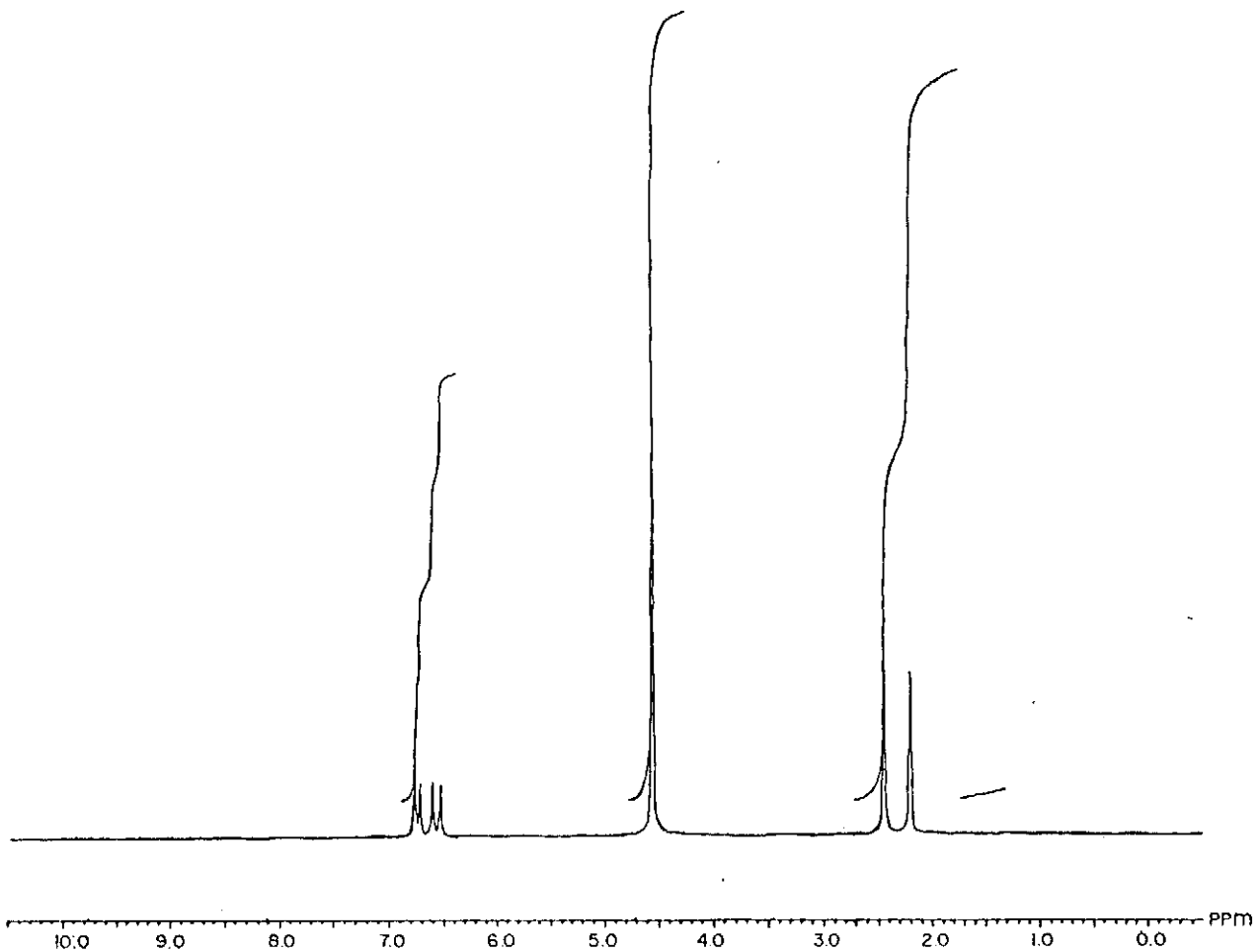


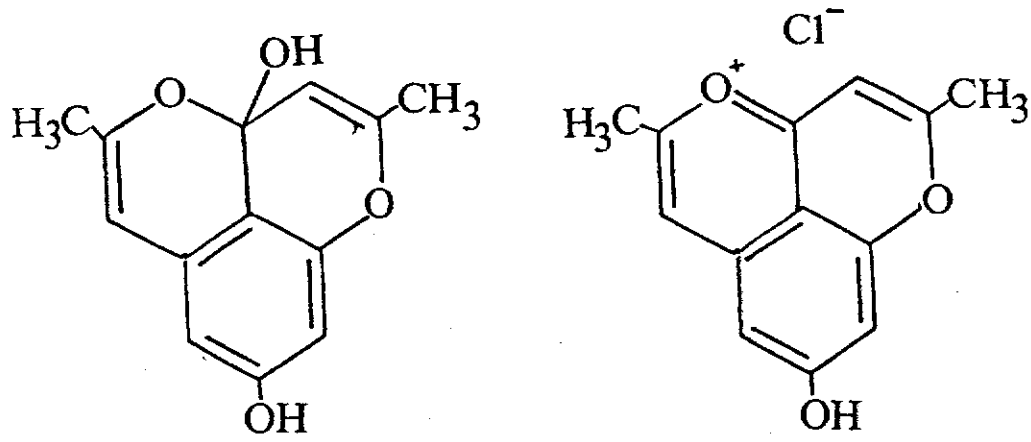
Figure 15 ^{1}H NMR spectrum of substance B.HCl, in D_2O (at 200 MH_Z).

Table 1 Spectroscopic characteristics of substance B and B.HCl compared with those described in previous work (Bycroft. 1970.).

Type of spectrum	substance B	Barakol (Bycroft. 1970.)	substance B.HCl	Anhydrobarakol hydrochloride (Bycroft. 1970.)
UV λ_{max} nm(logε)	in EtOH: 240(4.8) ,390(4.52)	in EtOH 241,246,384	in EtOH: 240(4.96) ,384(4.54)	in H_2O 241 and 472
IR ν_{max} (cm^{-1})	in KBr 3450, 1670, 1560, 1470	in Nujor 3450, 1670. 1630	in KBr 3500, 2740, 1660, 1442	in Nujor 3500, 3440. 1660. 1620
NMR δ value (ppm)	in $CDCl_3$+ DMSO-D_6, at 200MH_Z 5.81, 5.99 (2x1H,H-3,H-6) 6.00, 6.01 (2x1H,H-7,H-9) 1.94. 2.07 (6H,2xs,2xMe)	in $CDCl_3$, at 100 MH_Z 5.77, 6.03 (2 x 1H) 6.07, 6.17 (2 x 1H) 2.05, 2.16 (6H,2xs,2xMe)	in D_2O, at 200 MHz 6.80 (2x1H,H-9, H-7) 6.60(1H,H-6) 6.40(1H,H-3) 2.47,2.33 (6H,2xs,2xMe)	in D_2O, at 100 MHz 6.90 (4x1H) 2.48,2.70 (6H,2xs,2xMe)
Mass spectra (m/z)	M^+:232(>1),214(98) ,186(69),158(26) ,143(8),115(23), 93(20),89(16), 51(19),43(100)	M^+:232(2),214 (100),186(78), 158(23),143(6), 110(17),93(10), 89(10),51(13)	-	-

Identification of substance B and B.HCl as Barakol and Anhydrobarakol hydrochloride.

The chemical structure of the purified substance B and B.HCl were analyzed for physical and spectroscopic characteristics. It was found that those characteristics of the two products were correspond perfectly with barakol and anhydrobarakol hydrochloride.(Bycroft. 1970.)



Barakol **Anhydrobarakol hydrochloride**

Figure 16 Chemical structures of barakol and anhydrobarakol hydrochloride.

Comparison the data obtained from this study to those of Barakol and Anhydrobarakol hydrochloride (Bycroft. 1970.) confirmed that the two products are Barakol and Anhydrobarakol hydrochloride (Table 1).

2.Iodination reaction of anhydrobarakol hydrochloride.

The reaction between anhydrobarakol hydrochloride and sodium iodide using chloramine T as the oxidizing agent was found that at pH 2 , the reaction gave two iodinated compounds. The first obtained from precipitation as yellow brown crystals while the second obtained from the supernatant as yellow crystals. The yield of the two products are 50 and 28 percent respectively. At other pH, the reaction gave only yellow brown iodinated compounds. At pH 3 and pH 4, the reaction gave 36 and 33 percent yield respectively from precipitation and less than 10 percent from supernatant. At pH 5 and pH 5.89, the reaction gave approximately 18 and 17 percent yield respectively from precipitation. However, there was no substance found in supernatant. At pH 7.5, the reaction was unsuccessful due to the changing form of anhydrobarakol hydrochloride to barakol in mild basic condition.

The physical and spectroscopic characteristics of the substance A1 and A2 were evaluated. A substance A1 showed Rf value at 0.7 when using silica gel G60 F254 as the absorbent and the mixture of chloroform and methanol (9:1) as solvent system. Rf 0.25 when using the mixture of chloroform and ethyl acetate (6:4) as solvent system, and Rf 0.16 when using the mixture of chloroform and acetone (5:5) as solvent system. The physical and spectropic characteristics of the substance A1 was evaluated : mp. 220 °C (dec.) : UV λ_{max} (EtOH) nm(log ε) : 258(4.8) and 397(4.33) (Figure 17) : IR ν_{max} (KBr) 3427, 1664, 1565, and 1529 cm^{-1} (Figure 18) :

^{1}H-NMR ($CDCl_3$) : δ 6.69 (1H,s,H-6), 6.17(1H,s,H-3), 2.52, 2.30 (6H, 2xs,2xMe) (Figure 19) ; MS, *m/z* (rel.): M^+,468(2.7), 467(14.8), 466 (100),438(9), 339(17),311(15), 254(5), 212(3), 184(13), 169(12),156(4) , 144(6), 127(7),92(5), 64(4), 51(3), and 43(18) (Figure 20).

A substance A2 showed Rf value at 0.75 when using silica gel G60 F254 as the absorbent and the mixture of chloroform and methanol (9:1) as solvent system, Rf 0.3 when using the mixture of chloroform and ethyl acetate (6:4) as solvent system, and Rf 0.2 when using the mixture of chloroform and acetone (5:5) as solvent system. The substance A2 has melting point at 228 °C (dec.) ; UV λ_{max} (EtOH) nm(log ε) : 258(4.8) and 391(4.31) (Figure 21) ; IR ν_{max} (KBr) 3420, 1664, 1565, and 1520 cm^{-1} (Figure 22) ; ^{1}H-NMR ($CDCl_3$ + DMSO-d_6) : δ 6.69(1H,d,H-9), 6.67(1H,d,H-7), 2.49, 2.35 (6H,2xs,2xMe)(Figure 23) ; MS, *m/z* (rel.): M^+, 468(1.8), 467(14.9), 466(100),438(18), 311(16), 254(21),212(3), 184(14),169(13), 156(5), 144(6), 127(15), 92(6), 64(5), 51(4) and 43 (21)(Figure 24).

3. Verification of animal locomotion activity of anhydrobarakol hydrochloride and substance A1.

Anhydrobarakol hydrochloride significantly suppressed the locomotion activity of mice after intraperitoneal injection.The animal was observed in a sedative condition with seldom movement. The effect was clearly observed at approximately 2-3 minutes after injection and prolonged more than 90 minutes.

At dose 20 mg/kg body weight, the activity was frist observed to decrease (number of counts) at 5 minutes after injection and gradually down to the maximum decrease at 30 minutes and maintained until 90 minutes after injection(Figure 25). The decrease of activity was significantly difference from those of control during 5 to 90 minutes after injection. The mean activity during 120

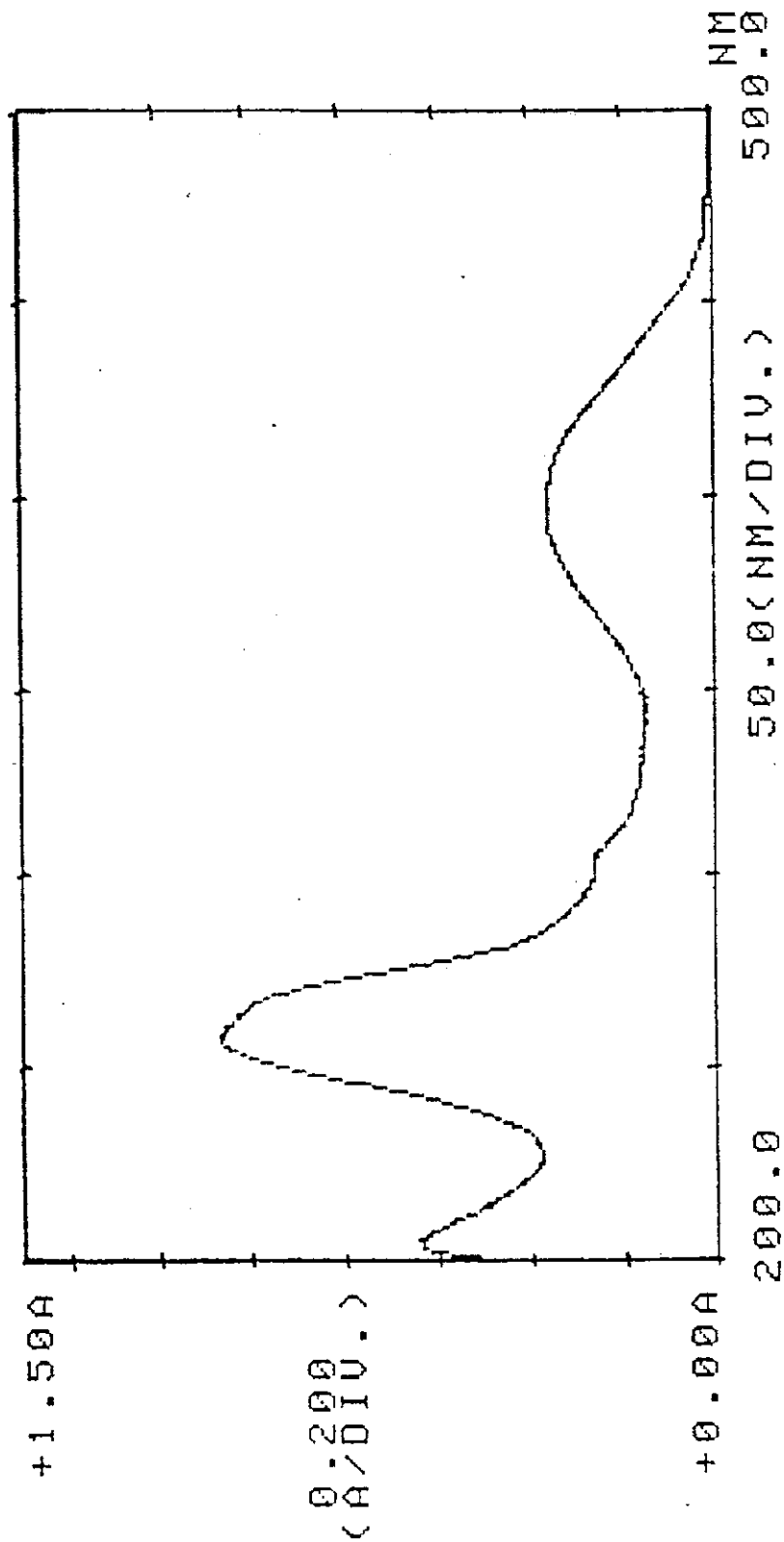


Figure 17 UV absorption spectrum of substance A1,in ethanol.

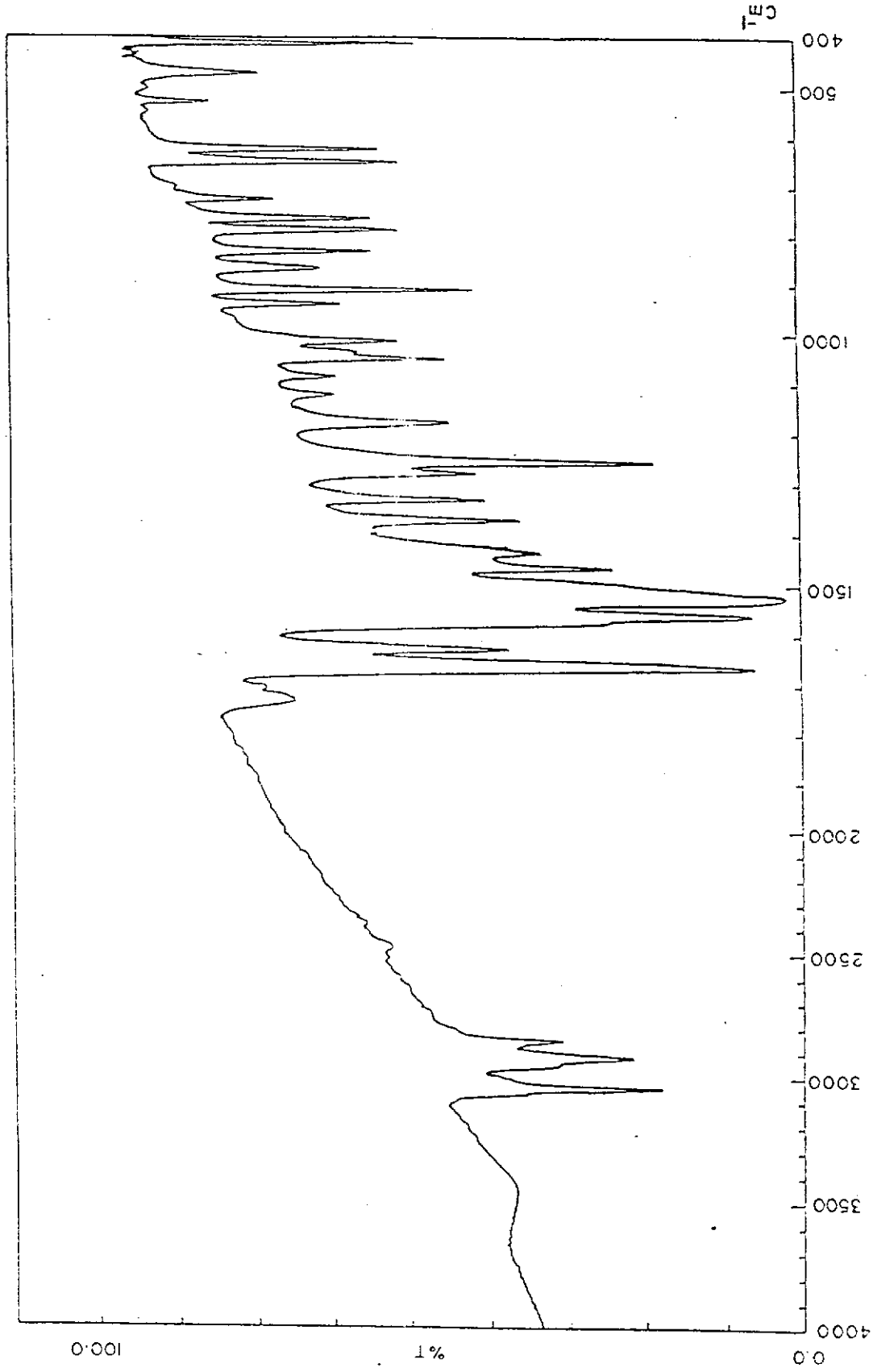


Figure 18 IR absorption spectrum of substance A1, in KBr.

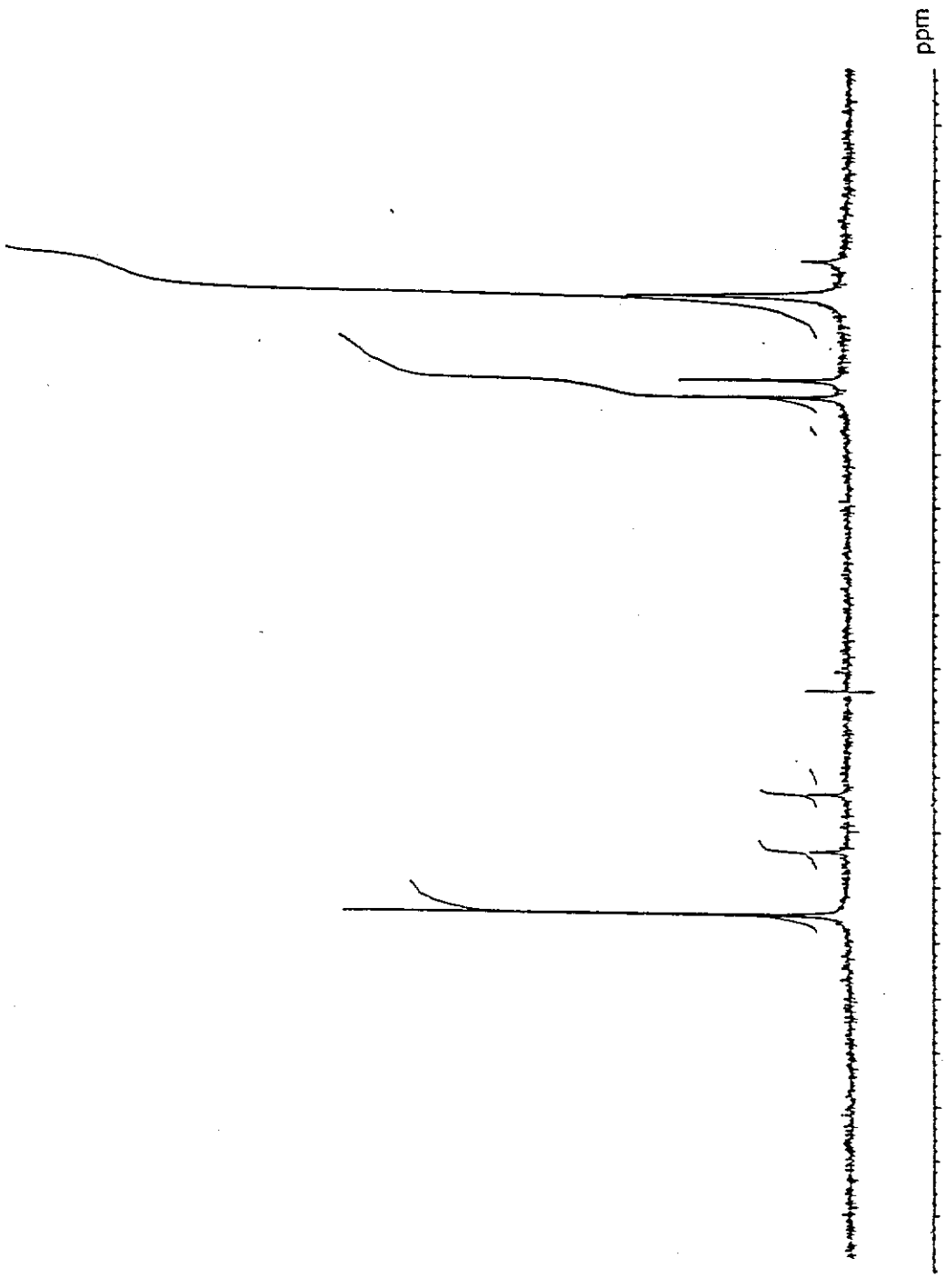


Figure 19 ^{1}H NMR spectrum of substance A1, in $CDCl_3$ (at 200 MH_Z).

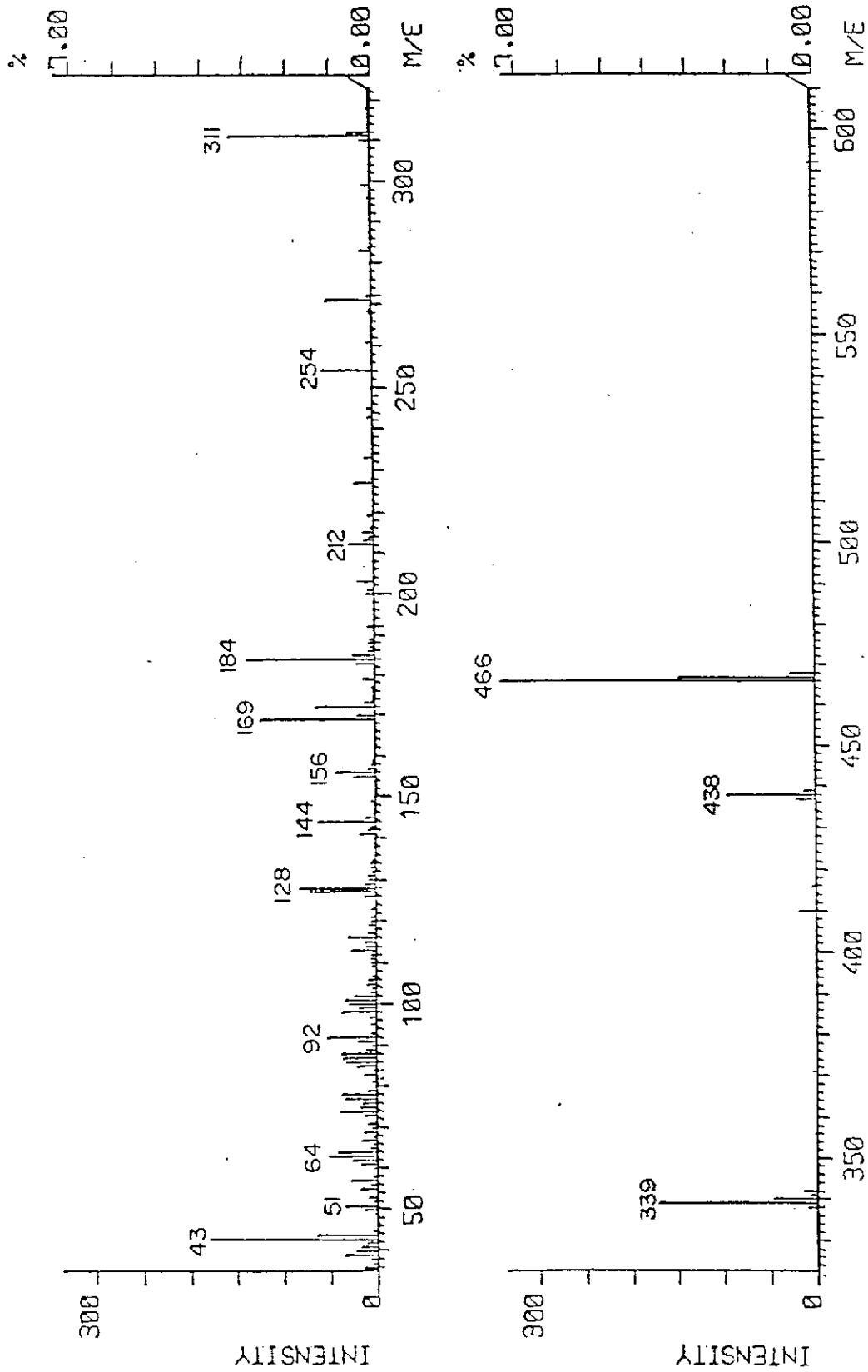


Figure 20 Mass spectra of substance A1.

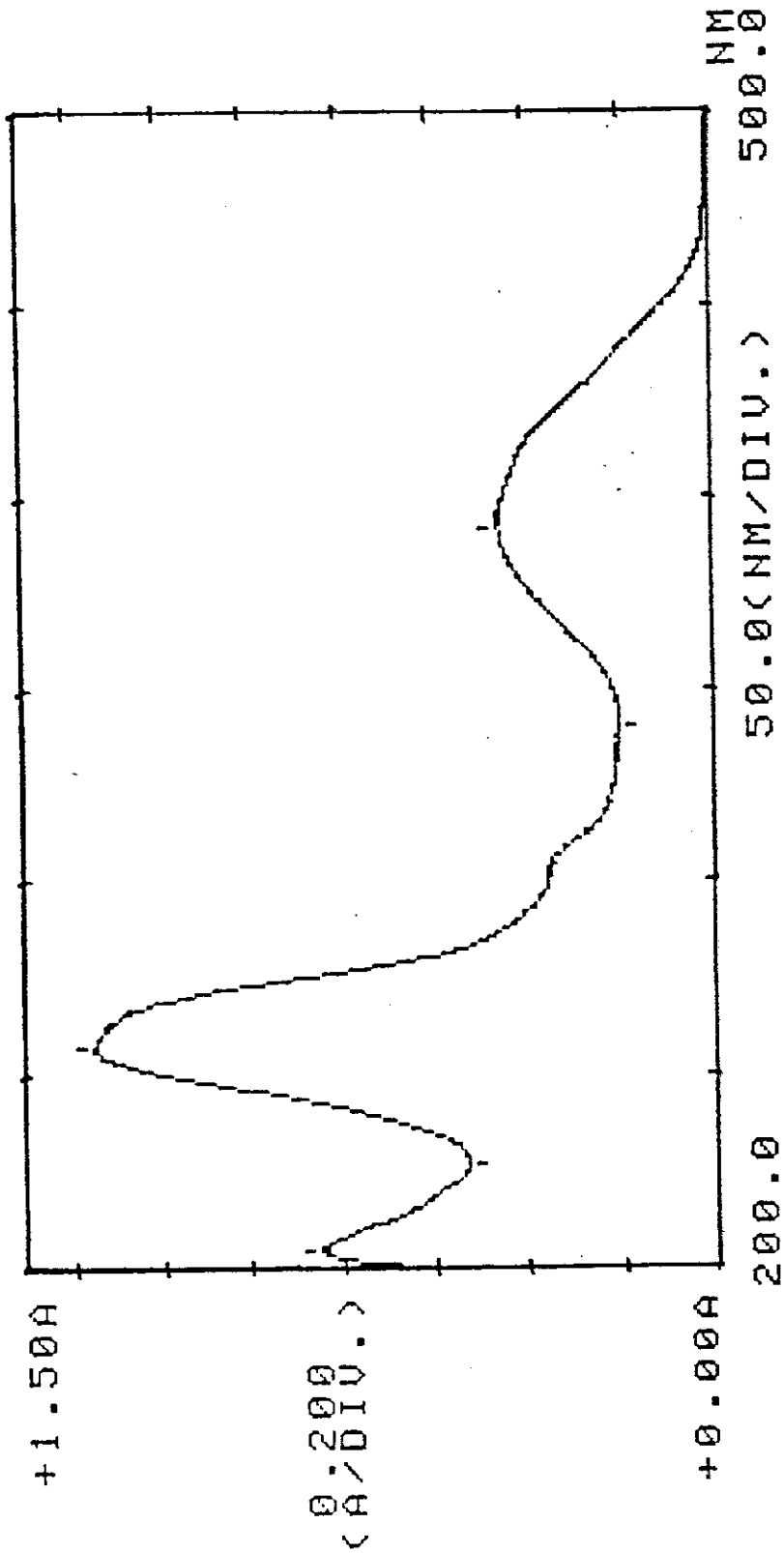


Figure 21 UV absorption spectrum of substance A2, in ethanol.

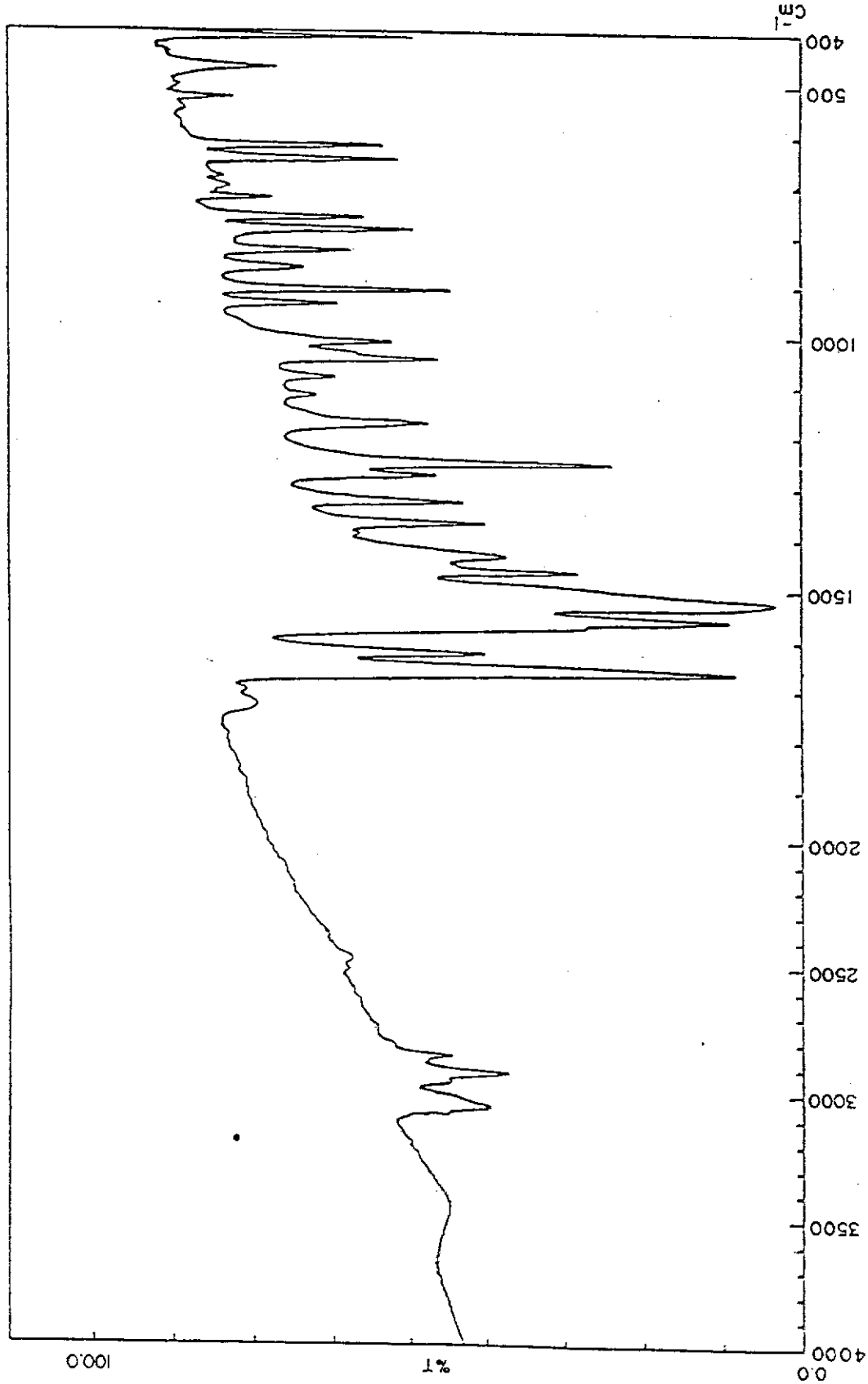


Figure 22 IR absorption spectrum of substance A2, in KBr.

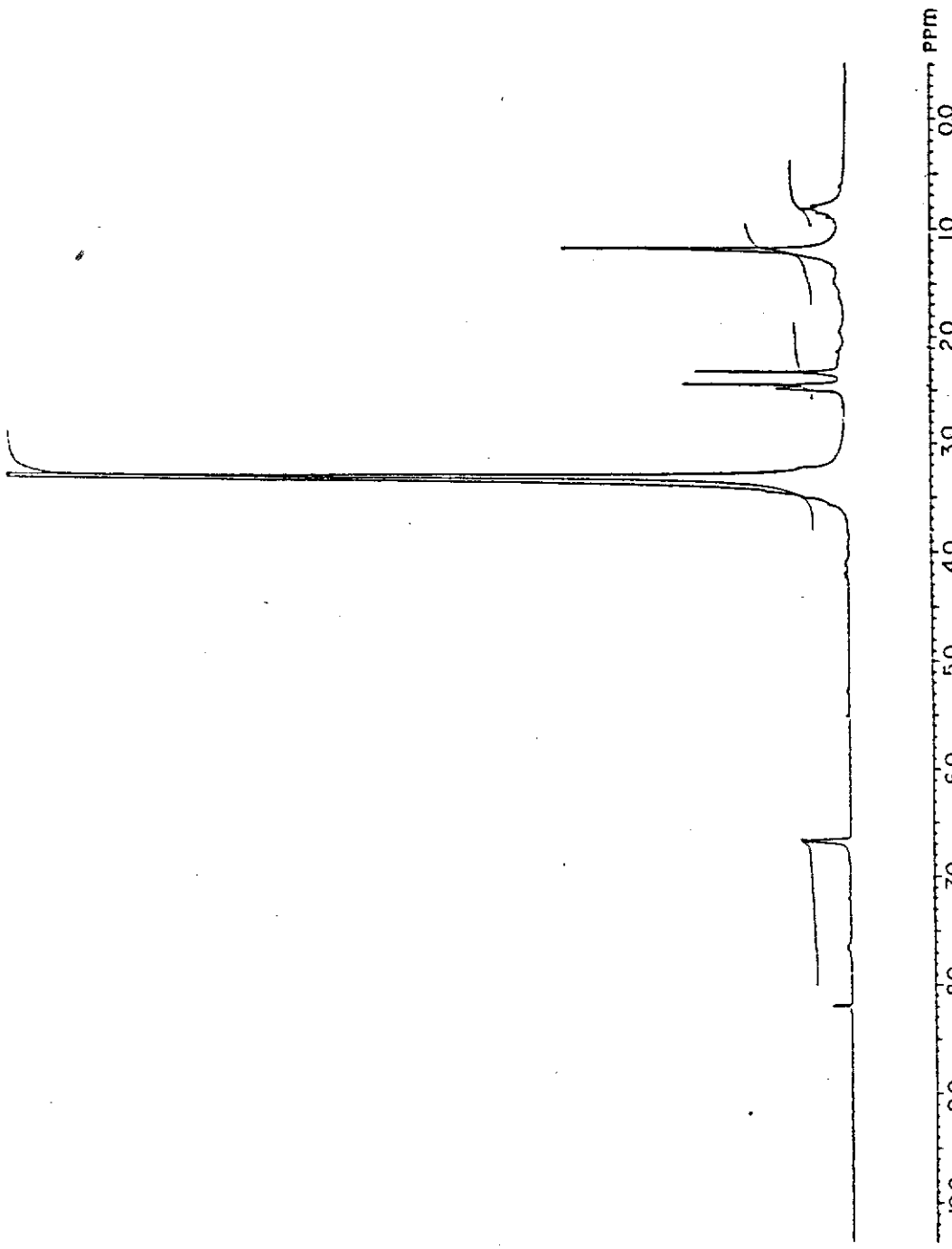


Figure 23 ^{1}H NMR spectrum of substance A2, in $CDCl_3$+ DMSO-d_6 (at 200 MH_Z).

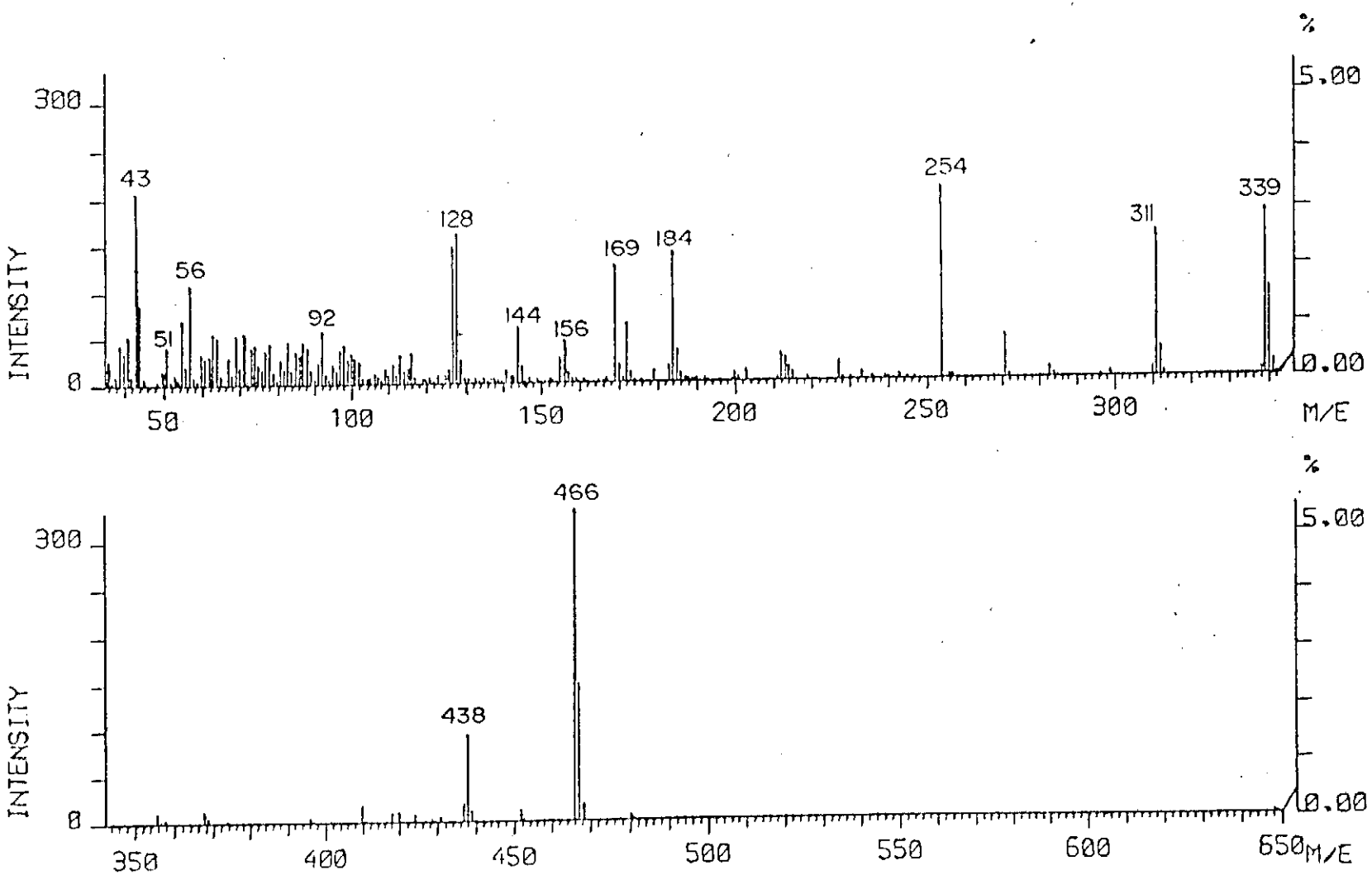


Figure 24 Mass spectra of substance A2.

minutes period after injection is 486.83 compared to 1274.00 of control. The percentage decrease is 61.80 (Table 2).

At dose 40 mg/kg body weight,the activity was frist observed to decrease (number of counts) at 5 minutes after injection and gradually down to the maximum decrease at 15 minutes and maintained until 90 minutes after injection(Figure 26).The decrease of activity was significantly difference from those of control during 5 to 90 minutes after injection. The mean activity during 120 minutes period after injection is 347.17 compared to 1195.80 of control. The percentage decrease is 71.00 (Table 2).

At dose 60 mg/kg body weight,the activity was frist observed to decrease (number of counts) at 5 minutes after injection and gradually down to the maximum decrease at 15 minutes and maintained until 90 minutes after injection(Figure 27). The decrease of activity was significantly difference from those of control during 5 to 90 minutes after injection. The mean activity during 120 minutes period after injection is 254.17 compared to 1089.2 of control. The percentage decrease is 76.70 (Table 2).

At dose 80 mg/kg body weight, the activity was frist observed to decrease (number of counts) at 5 minutes after injection and gradually down to the maximum decrease at 15 minutes and maintained until 105 minutes after injection(Figure 28).The decrease of activity was significantly difference from those of control during 5 to 105 minutes after injection. The mean activity during 120 minutes period after injection is 217.00 compared to 1140.50 of control. The percentage decrease is 81.00 (Table 2).

At dose 100 mg/kg body weight, the activity was frist observed to decrease (number of counts) at 5 minutes after injection and gradually down to the maximum decrease at 15 minutes

and maintained until 105 minutes after injection (Figure 29). The decrease of activity was significantly difference from those of control during 5 to 105 minutes after injection. The mean activity during 120 minutes period after injection is 173.83 compared to 1232.50 of control. The percentage decrease is 85.90 (Table 2).

The dose-response curve clearly demonstrated that the effect of anhydrobarakol hydrochloride depend on its dose (Figure 30). The more dose used, the stronger effect, the effect gradually increased to in relation to the increase of the dose.

The iodinated product(A1) also decreased the locomotion activity of experimental mice 60 mg/kg body weight ; the decrease of activity was frist observed at 5 minutes after injection and gradually down to the maximum decrease at 15 minutes and maintained until 60 minutes (Figure 31). The decrease during the period 5 to 60 minutes is significantly difference from those of control ($P < P_{0.05}$). The mean activity during 5 to 120 minutes period is 632.67 compared to 1316.00 of control. The percentage decrease is 51.90 and this difference is significantly (Table 2). The effect of the A1 is less than that of anhydrobarakol hydrochloride at the same dose (Figure 32), (Table 3).However, the difference of the effect between these two compounds are non significantly.

Table 2 Effect of B.HCl (anhydrobarakol hydrochloride) at various dose on mice locomotion activity.

Dose (mg/kg)	Normal Activity (number of counts)	Test Activity (number of counts)	% Decrease of Activity	Willcoxon pair test ($N=6, P_{0.05}$)
B.HCl				
20	1274.00	486.83	61.80	significant
40	1195.80	347.17	71.00	significant
60	1089.20	254.17	76.70	significant
80	1140.50	217.00	81.00	significant
100	1232.50	173.83	85.90	significant
A1				
60	1316.00	632.67	51.90	significant

Table 3 Comparison of the locomotion activity in mice after receive B.HCl and A1 at 60 mg/kg body weight.

Substance dose=60 mg/kg	Normal Activity (number of counts)	Test Activity (number of counts)	%Decrease of Activity	Mannwhitney U'test ($N=6, P_{0.05}$)
B.HCl	1089.17	254.17	76.70	Non sign.
A1	1316.00	632.67	51.90	

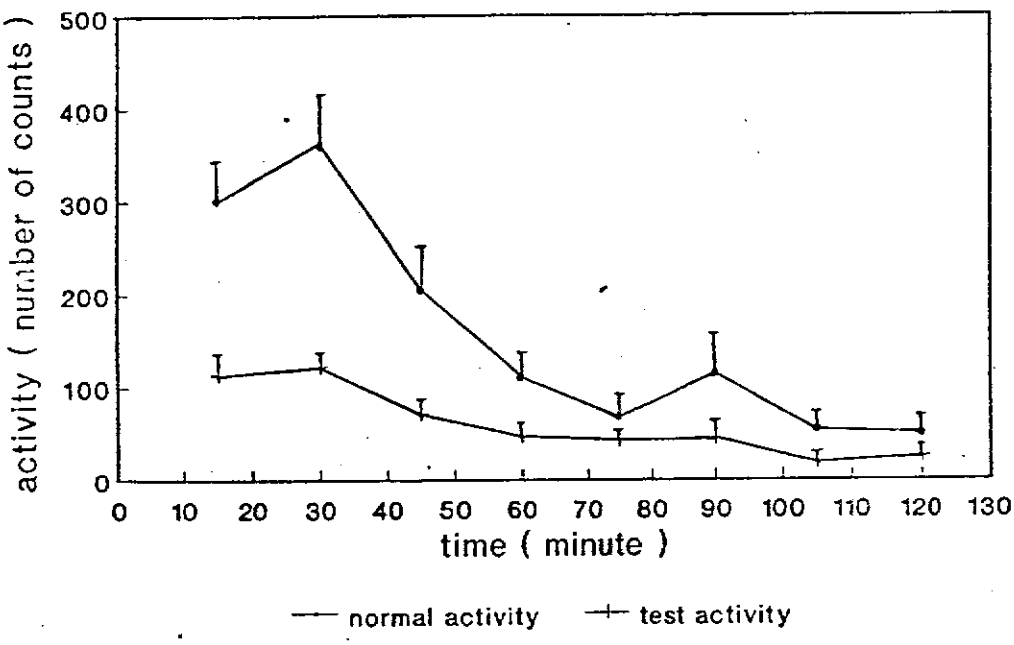


Figure 25 Time response curve show locomotor counts of mices (mean + S.E.M.) after treatment with normal saline as normal activity (control group) and substance B.HCl dose 20 mg/kg as test activity (test group).

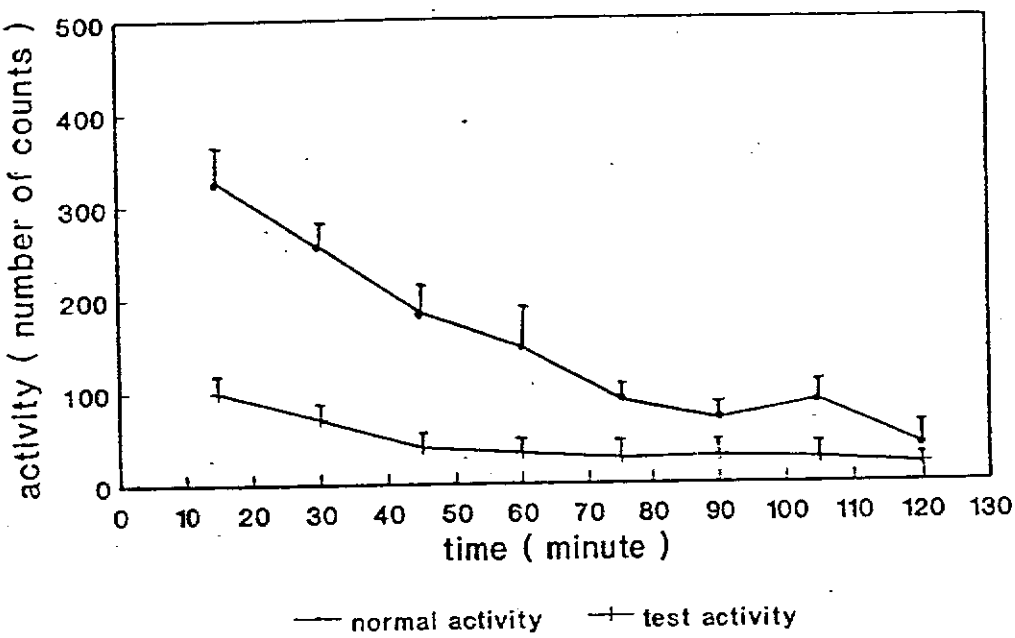


Figure 26 Time response curve show locomotor counts of mices (mean + S.E.M.) after treatment with normal saline as normal activity (control group) and substance B.HCl dose 40 mg/kg as test activity (test group).

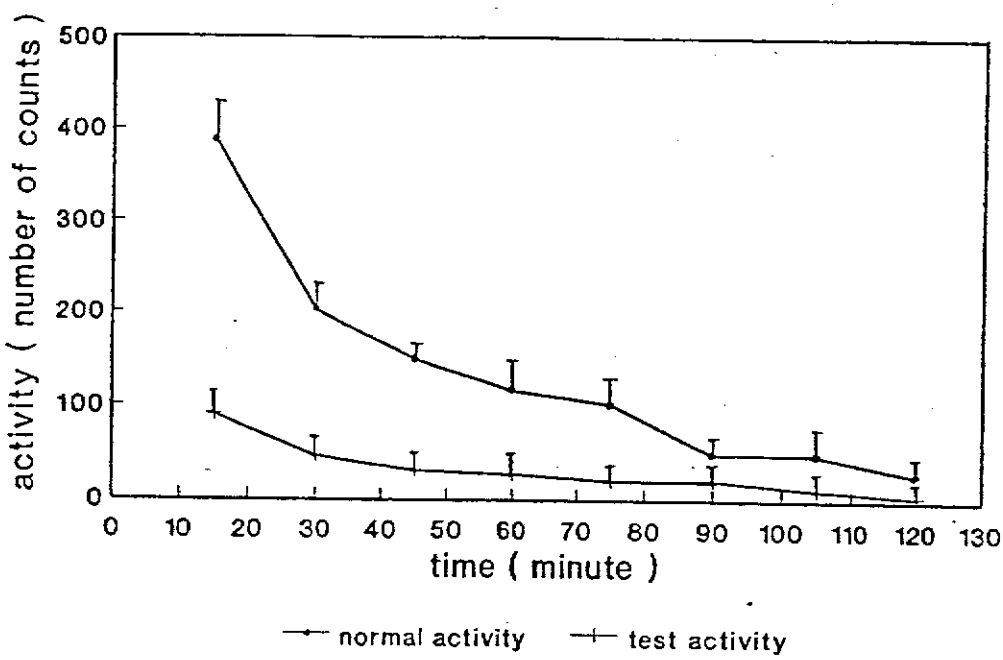


Figure 27 Time response curve show locomotor counts of mices (mean ± S.E.M.) after treatment with normal saline as normal activity (control group) and substance B.HCl dose 60 mg/kg as test activity (test group).

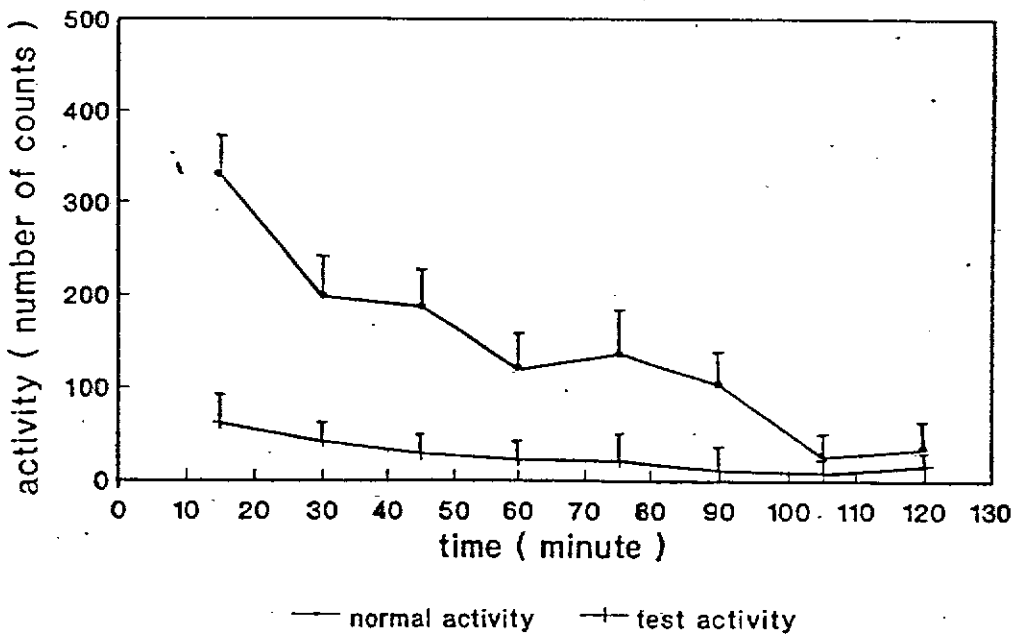


Figure 28 Time response curve show locomotor counts of mices (mean + S.E.M.) after treatment with normal saline as normal activity (control group) and substance B.HCl dose 80 mg/kg as test activity (test group).

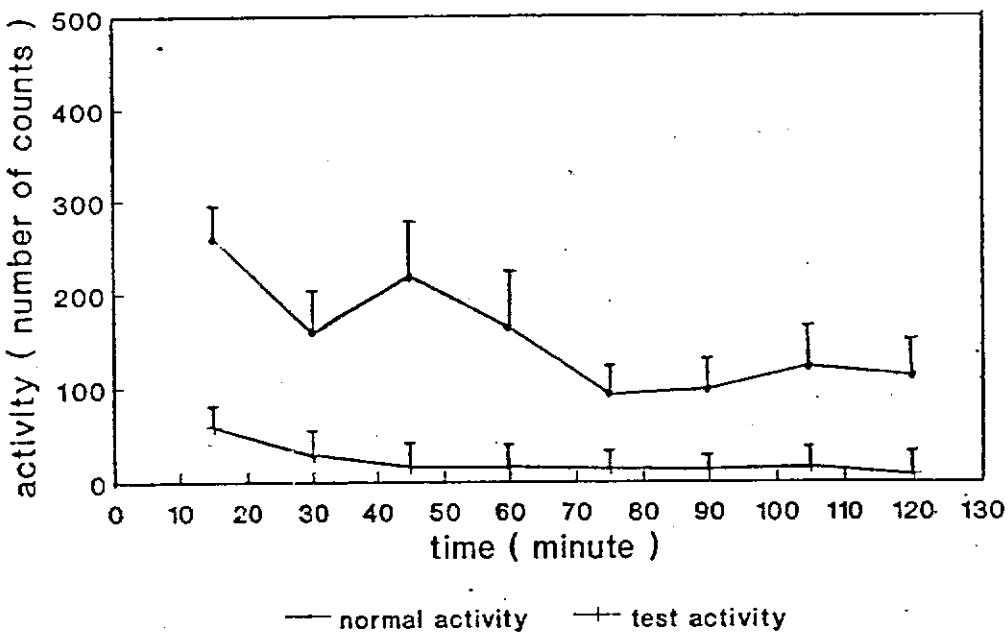


Figure 29 Time response curve show locomotor counts of mices (mean + S.E.M.) after treatment with normal saline as normal activity (control group) and substance B.HCl dose 100 mg/kg as test activity (test group).

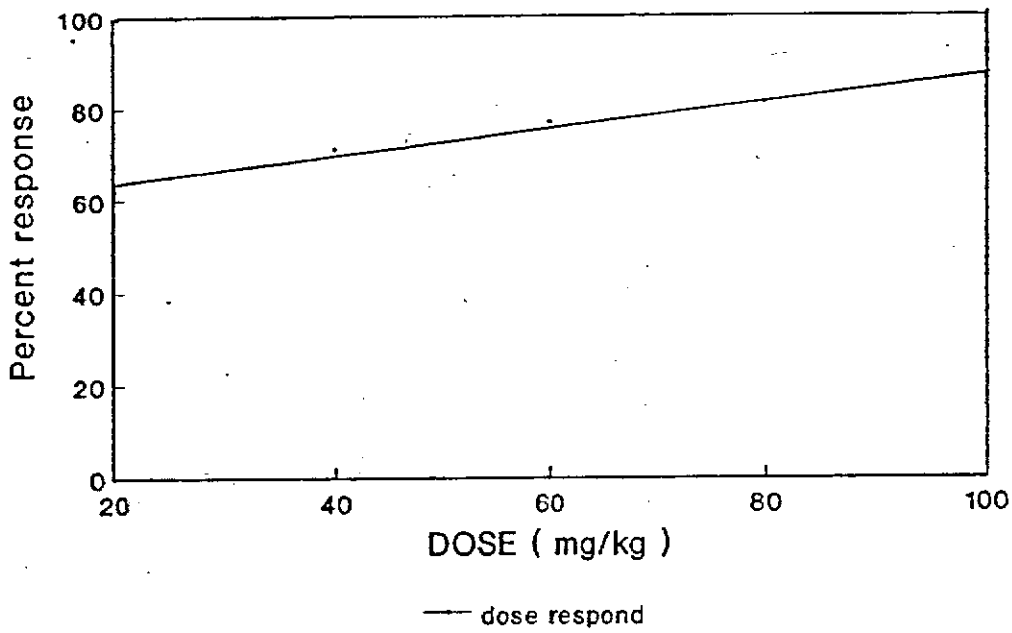


Figure 30 Dose response curve of % decrease of activity after treatment with substance B.HCl dose 20, 40, 60, 80, and 100 mg/kg.

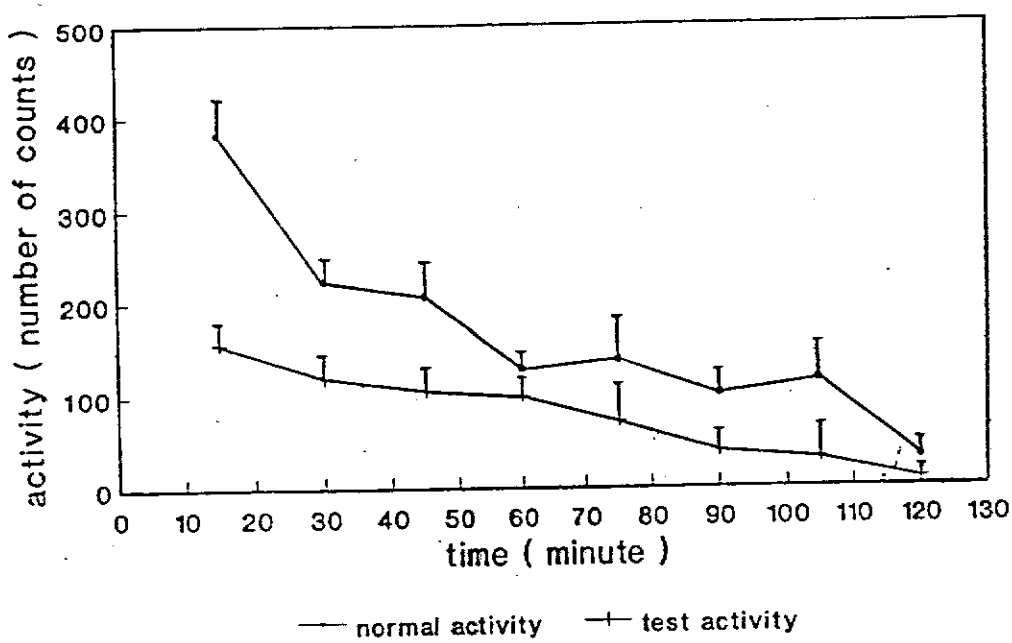


Figure 31 Time response curve show locomotor counts of mices (mean + S.E.M.) after treatment with normal saline as normal activity (control group) and substance A1 dose 60 mg/kg as test activity (test group).

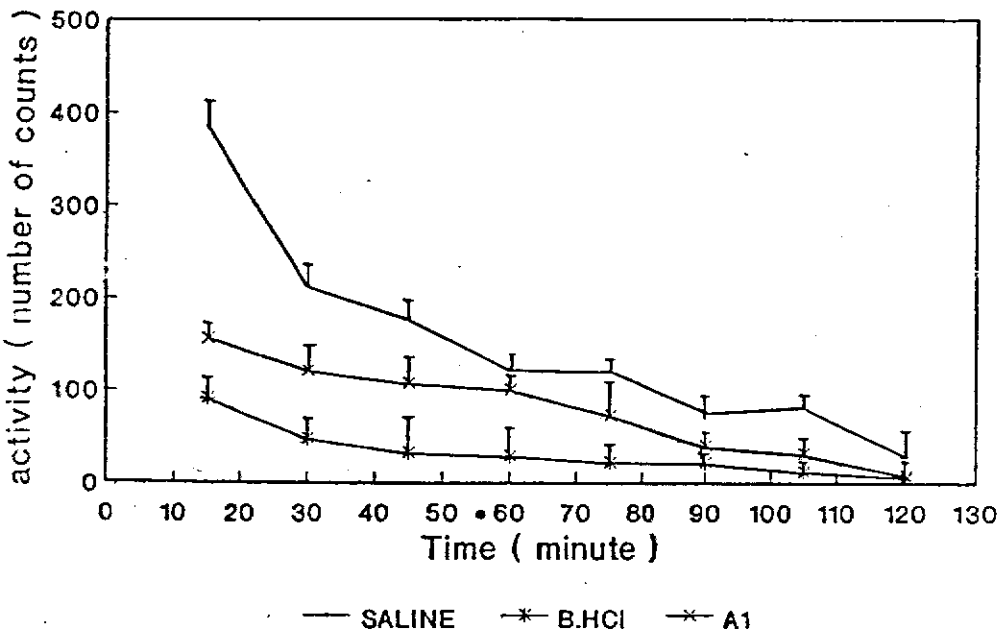


Figure 32 Time response curve show locomotor counts of mices (mean + S.E.M.) after treatment with normal saline as normal activity (control group) compairing with substance B.HCl and A1 dose 60 mg/kg as test activity (test group)

CHAPTER IV

DISCUSSION

Preparation of Barakol.

Barakol was extracted from the fresh young leaves of *Cassia siamea* Lamk. by boiling twice the plant materials with 0.5 % sulphuric acid followed by alkalinization with sodium hydrogen carbonate. These reactions offered 0.3 % yield which is much more than those obtained from the previous technique (Hassanali, 1969., Chaichantypyth. 1979.) which gave only 0.02 and 0.1 % yield respectively. The improvement of the yield may be result from the mild conditions of acid (0.5 % H_2SO_4) and base ($NaHCO_3$) during extracted employed in our study. Conversely, the previous techniques employed stronger than condition of acid (1%CH_3COOH) and base (25 % NH_3 solution). This seems to convince that the optimal condition of reaction for the extraction barakol should be mild.

Iodination reaction of Anhydrobarakol hydrochloride.

It was quite obvious that the iodination reaction of anhydrobarakol hydrochloride will be taken place when the chloramine T is the oxidizing agent at pH 2, 3, 4, 5, and 5.89. However, only reaction at pH 2 yield two iodinated products (A1 and A2) while at the other pH, the reaction yield only the A1 product. The pH 2 was shown to be the optimal for the reaction giving the highest yield of A1 = 50 %, A2 = 28 % to 36, 33, 18, and 17 % obtained at pH 3, pH 4, pH 5 and pH 5.89 respectively. (Figure 33).

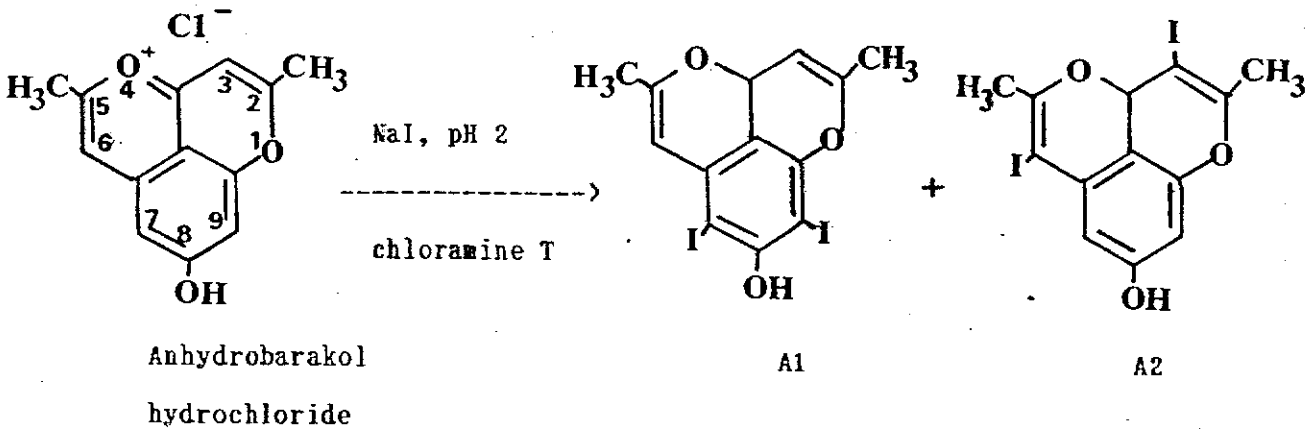


Figure 33 Iodination reaction of anhydrobarakol hydrochloride.

High resolution mass spectrometry demonstrated that the weak peak at *m/z* of A1 and A2 are 468. This molecular weight indicated the substitution of two iodine atoms and loss of two hydrogen atoms from anhydrobarakol hydrochloride molecule since the molecular weight of anhydrobarakol hydrochloride molecule is 215 (excluding mass unit of Cl^-), two iodine atoms are 254 and two hydrogen atoms are 2.

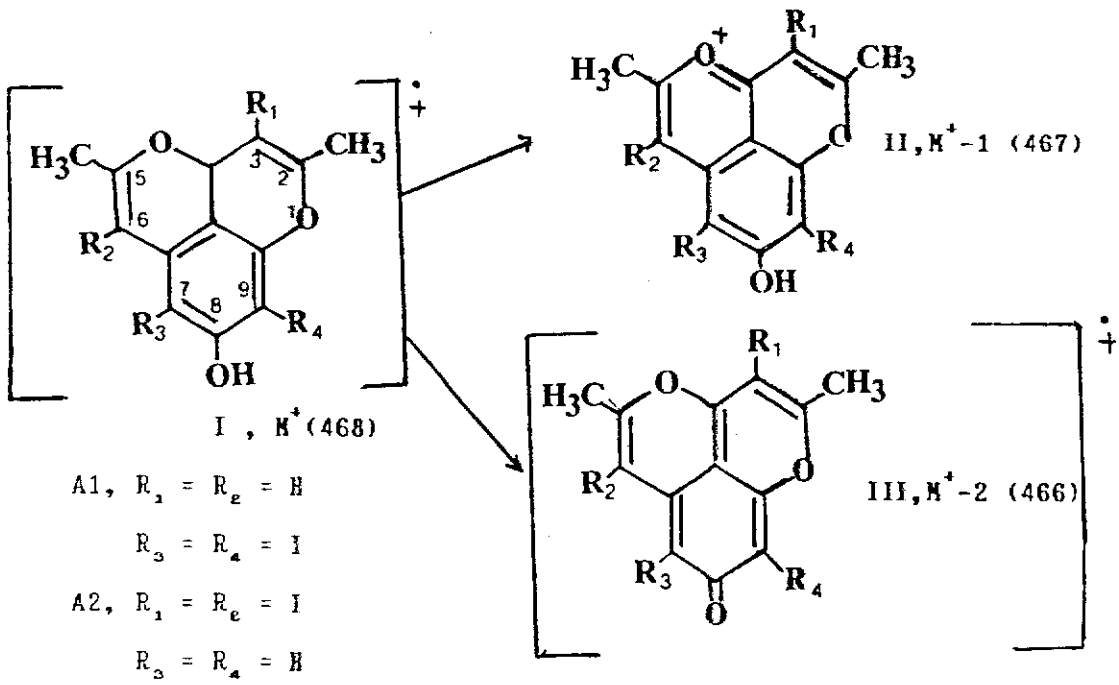


Figure 34 Conversion formation of the product from iodination reaction.

The molecular formula of A1 and A2 ($C_{12}H_{10}I_2O_3$) emerged from high-resolution mass-spectrometry(Figure 20 and 24). Other interesting feartures of the mass-spectrum of A1 and A2 are at 467 *m/z* it show the salt formation in structure II (Figure 34) and the mass-spectrum are a base peak at 466 *m/z* corresponding to the loss of hydrogen atom at 3a and hydrogen atom of hydroxyl group at 8 positions (Figure 34).

The UV spectrum of A1 and A2 show both λ maxima at 258 nm which is the characteristics of chromone type- structure (Wagner. 1978.). This can be concluded that the A1 and A2 must have chromone type- structure. The IR spectrum of A1 and A2 exhibit strong bands at 1664 cm^{-1} which are assigned to a hydrogen-bound carbonyl group as in barakol and anhydrobarakol hydrochloride.

The ^{1}H-NMR spectrum of substance A1 (in $CDCl_3$) show two methyl protons signals at δ 2.52 and 2.30 and two vinyl protons signals at δ 6.17 and 6.69. The ^{1}H-NMR spectrum of the barakol demonstrated two methyl protons signals at δ 1.94 and 2.07 , two vinyl protons signals at δ 5.81 and 5.99, two meta- coupling aromatic protons signals at δ 6.00 and 6.01. The disappearance of the two - meta coupling aromatic protons signals suggested the two iodine atoms substituted these two aromatic protons. Thus , all spectroscopy spectra confrims the structure of A1 as iododerivative of barakol (Figure 33). The substitution of iodine molecule took place at 7 and 9 positions of barakol.

The ^{1}H-NMR spectrum of substance A2 (in $CDCl_3$ + DMSO-d_6) show two methyl protons signals at δ 2.35 and 2.49 and two meta-coupling aromatic protons signals at δ 6.67 and 6.69. The ^{1}H-NMR spectrum of the barakol demonstrated two methyl protons signals at 1.94 and 2.07, two vinyl protons signals 5.81 and 5.99 , two meta-

coupling aromatic protons signals at δ6.00 and 6.01. The disappearance of the two vinyl protons signals suggested the two iodine atoms substitution these two vinyl protons (Figure 34).

In organic solvent ($CDCl_3$ and DMSO-d_6) A1 and A2 must be in structure III (Figure 34) because of the ^{1}H-NMR spectrum of their show clearly the disappearence of hydrogen atom at 3a and hydrogen atom of hydroxyl group at 8 positions. This is also corresponding to the mass-spectrum (of A1 and A2) are a base peak at 466 *m/z* (Figure 20,24 and 34). Thus, the structure III (Figure 34) could be the stable form of A1 and A2 .

The substitution of iodine atoms at 7 and 9 positions of barakol is the same as those radioiodination reactions of the proteins containing tyrosine receduce. (Bolton. 1989.) This suggested that the mechanisms underlied these two categories of reactions should be similar. However,the former reaction gave much lower yield (50 %) than those of the latter one (80 %). This seems to be due to acid -base influence phenomenon. The iodination reaction of tyrosine takes place at mild basic condition (pH 7-8) (Due. 1964.) which induce electron delocalization from hydroxyl group to both ortho positions in the phenolic ring. This activate the proton in the ortho position labile for electrophilic attack such as iodine (Figure ; Due. 1964. , Seon. 1970.) However, the iodination reaction of anhydrobarakol hydrochloride was confined to pH 2 due to the transformation of anhydrobarakol hydrochloride to barakol at higher pH conditions. The barakol will be precipitated and make the reaction impossible. Thus, under pH 2 the delocalization of electron from the hydroxyl group was inhibited due to the unability of group to ionize. Thus, the ortho positions are not labile to the electrophilic attack. The iodine substitution of A2 at 3 and 6 of barakol which is different

from those of A1. The 3 and 6 positions are also labile to the electrophilic attack due to delocalization of electrons from the methyl groups to the ring. However, this suitability took place harder than those of 7 and 9 positions of barakol (Grovenstein. 1962.).

Verification of animal locomotion activity.

In this study it was found that the barakol significantly induce the sedative action in experiment in every doses . This confirmed the previous report (Jantarayota. 1989.). The anhydrobarakol hydrochloride induces the effect within 5 minutes after injection. This induction time is rather short which indicate the effectiveness of the substance.

The duration of action of anhydrobarakol hydrochloride is related to the dose. At lower doses (20-60 mg/kg) the duration is approximately 90 minutes which at higher doses is 105 minutes. Likewise, the strength of the action is also related to the dose. The increase of the effect is linear from dose 20-100 mg/kg body weight. The experimental animals did not show any abnormal sign except. This suggested that all doses used in this study seemed to be quite safe.

The iodinated product A1 also induced sedative effect in experimental animals. The effectiveness of the substance seemed to be the same as anhydrobarakol hydrochloride since the reaction time is the same (5 minutes). A1 induced the effect only 60 minutes period which is shorter than that anhydrobarakol hydrochloride at the same dose. Likewise, the strength of the action is also reduced the locomotion activity 51.90 percent when compared to 76.70 percent of the anhydrobarakol hydrochloride. This results indicated that iodine substitution slightly changed the anhydrobarakol hydrochloride configuration and the main configuration still remained the same. However, the decrease of the activity induced by the A1 (51.90 %) is

not significant difference from those of anhydrobarakol hydrochloride (76.70 %). This suggested that iodinated product of the anhydrobarakol hydrochloride will retain biological activity and the reactive site is still intact as the precursor. This confirm the possibility of using the iodinated product as the radiotracer for the receptor study of the barakol in the central nervous system.

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การศึกษาปฏิกิริยาไฮโดรเจนของสารบาราคอลที่สกัดได้จากใบอ่อนของต้นชี้เหล็ก และการทดสอบฤทธิ์ในการลงประจับ

บทคัดย่อ

ของ

กฤษณา เกาะแก้ว

เสนอต่อมหาวิทยาลัยศรีนครินทรวิโรฒ ประสานมิตร เพื่อเป็นส่วนหนึ่งของการศึกษา
ตามหลักสูตรปริญญาวิทยาศาสตร์มหาบัณฑิต สาขาเคมีชีวภาพ
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บทคัดย่อ

บาราคอล (3a, 4- dihydro- 3a, 8- dihydroxy-2, 5-dimethyl-1, 4-dioxaphenalene) เป็นสารที่ได้จากปฏิกิริยาของ 5-acetonyl-7-hydroxy-2-methylchromone กับกรด และสามารถสกัดได้จากใบสดอ่อนๆของต้นชี้เหล็ก เกลือคลอไรด์ของบาราคอลคือ แอนไฮโดรบาราคอลไฮโดรคลอไรด์ แสดงฤทธิ์เบื้องต้นในทางสงบระงับในหนูขาว จากการทำปฏิกิริยาไอโอดิเนชันของแอนไฮโดรบาราคอลไฮโดรคลอไรด์โดยใช้รีเอเจนไอโอไดด์ และมีคลอรามีนทีเป็นตัวออกซิไดซ์ ในสารละลายกรด ที่pH 2 ให้ผลิตภัณฑ์ 2 ชนิด ผลิตภัณฑ์หลักที่ได้มีไอโอดีน 2 อะตอม เข้าแทนที่ที่ตำแหน่ง 7 และ 9 ส่วนสารผลิตภัณฑ์รองไอโอดีน 2 อะตอม เข้าแทนที่ที่ตำแหน่ง 3 และ 6 ในโมเลกุลของบาราคอล

เมื่อทำการทดสอบฤทธิ์เบื้องต้นในทางสงบระงับของสารผลิตภัณฑ์หลัก พบว่าขนาด 60 mg/kg สามารถลดการเคลื่อนไหวของหนูขาวไปได้ในปริมาณร้อยละ 51.9 หรือคิดเป็นฤทธิ์ในทางสงบระงับเหลืออยู่ประมาณ 68% เมื่อเทียบกับผลที่ได้จากแอนไฮโดรบาราคอลไฮโดรคลอไรด์ในขนาดเดียวกัน ดังนั้นแนวทางในการเตรียมสารบาราคอลเพื่อใช้เป็นสารติดตามติดฉลากไอโอดีนไอโซโทป เพื่อใช้ในการศึกษากลไกการออกฤทธิ์ของบาราคอลในสมองโดยเทคนิค Receptor Autoradiography มีทางเป็นไปได้

IODINATION REACTION AND EVALUATION OF SEDATIVE ACTION OF BARAKOL, THE MAIN INGREDIENT EXTRACTED FROM THE YOUNG LEAVES OF CASSIA SIAMEA LAMK.

AN ABSTRACT

By

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September 1992

ABSTRACTS

Barakol (3a, 4-dihydro- 3a, 8-dihydroxy- 2, 5-dimethyl- 1, 4-dioxaphenalene) is the reaction product obtained from the acid treatment of the natural occurring methyl chromone (5-acetonyl-7-hydroxy-2-methylchromone) extracted from fresh young leaves of *Cassia siamea* Lamk. Its hydrochloride salt effectively induces sedative effect in experimental mice. Reaction of the substance with sodium iodide and chloramine T as the oxidizing agent in hydrochloric acid solution (pH 2) gave two iodinated products. The major one has two iodine atoms replaced two hydrogen atoms of the carbons at 7 and 9 positions of the barakol. While the minor product has two iodine atoms replace the hydrogen atoms of carbons at 3 and 6 positions.

The major product was also found to induce sedative effect in experimental mice at 68 percent when compared to barakol treatment at the same dose. Thus, this findings suggested the possibility of preparing radiotracer from the barakol for the detailed study of the mechanism of the actions of the barakol by the receptor autoradiography.