THE ASSESSEMENT OF SOME BIOLOGICAL ACTIVITIES AND EXCRETORY ROUTES OF AN UNKNOWN COMPOUND. FK-

BY

MARIGAH J.

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A C K N O W L E D G E M E N T S

I would like to thank my Supervisor, Dr. G. Muriuki for the invaluable help and encouragement he gave me. Also I wish to thank all those without whom this could not have been.

A B S T R A C T :

The purpose of this project was to investigate the biological activities of a synthetic compound and whether any of its metabolic products are detectable in urine and bile. Investigations were also carried to find out whether the compound was modified while in blood.

The synthetic compound was thought to be a non-steroidal anti-inflammatory agent. Analgesic and diuretic activities of the agent were also investigated.

The results showed that the agent had some analgesic and diuretic activities. Its metabolites were detectable in urine and bile. Blood samples obtained from the rabbits after intravenous injection of the agaent showed that the compound was not modified in any was while in blood.

In the experiments carried out, Digoxin, Sulidac and Nutmeg suspension were used to find out the suitable soluent and visualising methods which would be used to detect the metabolites in urine and bile.

The standardizing drugs are known to be excreted through bile and urine. Cannulation techniques were employed to obtain bile samples that were to be screened for the presence of the metabolites.

This layer chromatographic techniques were used in detection of the metabolites.

In screening for analgesic activity on a hot plate which was maintained at a temperature of 50-55^{o}C and analgesiometer were used.

INTRODUCTION

Drugs give relief to pain in two ways:-

Firstly they reduce the ability of the patient to perceive the sensation probaly by an action on the thalamus or by altering the appreciation of the senation so that it is no longer unplesant, probably by an action of the cerebral cortex.

The pain threshold itself is influenced by the emotional state of an individual, eg, the pain that a patient beleives to be a "heart attack" may cause greater distress than pain of same intensity in a leg.

The quantitative estimation of analgesia is very difficult and it cannot be known for sure if a noxious stimulus applied to an animal approximates the nervous stimulus of pain experienced by man. Certain qualities of pain are not easily reproduced experimentally and some analgesics often fail to show any analgesic activity experimentally.

Some anti-inflammatory drugs like the salicylates may play a role in relieving pain. Though assessement of analgesia is very difficult, hot plate method is often used to assess it.

A compound is classified as a diuretic if it causes an increase in output of sodium and water. Their main effect is on the nephron (the proximal tubule, the loop of Heult and the distal tubule). They interfere with the re-absorption mechanisms of Sodium ions. The high concentration of the Sodium ions in the tubule will alter the consentration of the tubule causing water be withdrawn into the tubule to balance the hypertonic solution. This will lead to production of large volume of dilut urine.

Drugs are biotransformed through various metabolic pathways, eg, Hydroxylation, N-Methylation, Deamination, reduction etc.

Once the drug is biotransformed, it is conjugated to form a more polar derivative which is rapidly eliminated than the parent compound. Though in few cases more toxic product may be produeed, metabolism often results in a less toxic product. The major sites of drug biotransformation are the liver, the kidneys, GIT and plasma.

excreted from the body.

The excretory routes are several but the major ones include renal, and biliary. Renal route is by far the most important route for disposition of foreign compounds. For a compound to be excreted through the renal route, it must be water soluble and have a low molecular weight such that it can be filtered into the glomerular from the blood stream, and should not be protein-bound.

Biliary route is important in the excretion of compounds with high molecular weights. They are usually anions, cations and non-ionised molecules containing both polar and lipophilic groups. Rate of biliary secretion may drastically be reduced in liver damage.

The purpose of the present study was to investigate whether the unknown compound (FK-) had any pharmacological activity on whole animal and whether biotransformed in the body, any of the metabolit could bedetected in bile and in urine. The study was carried out under four broad headings:-

i) Demonstration of biological activity in a whole animal.
ii) Investigation of any analgesic activity.
iii) Investigation of any diuretic activity.
iv) Detection of any metabolites in urine and bile.

EXPERIMENTAL

I) OBSERVATION IN WHOLE ANIMAL:

A group of four mice of average weight of 25 grams were selected Two of these mice were injected with 0.6mg per 100g body weight intraperitoneally with the synthetic compound.

The remaining two mice were injected intraperitoneally with water. These were the control.

After injection of the compound, the time clock was started and after every 2-minutes any changes in the following were observed.

1) Reflexes: The righting, the pedal, and the corneal reflexes.
ii) Cardio-vascular system: Whether there was an increase or decrease on heart-rate.
iii) Central Nervous System: The effect of the compound on the central nervous system was also investigated. This involved checking whether the compound caused any drowsiness, restlessness or excitability in the animal. Depression was assessed by noting whether there was any muscular inco-ordination together with loss of righting reflexes.
iv) Respiratory System: The effect of the drug on the respiratory system was also noted. This involved checkinh whether the breathing rate of the animal was changed in any way; whether the breathing was deep or shallow.

The Observation were recorded every two to three minutes for one and a half hours. The observations were compared with the control group which was injected intraperitoneally with saline.

II) ANALGESIC ACTIVITY

A group of six mice were selected. Only those mice which initially reacted within 30 seconds were used in the experiment.

Each mouse was injected intraperitoneally with 0.6mg/100g body weight with the unknown drug; Pethidine (10mg/kg); and Sulidac (2mg/k

The analgesic activity was carried out using (i) the hot plate and (ii) the analgesiometer.

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i) The Hot Plate Method

Once the mouse was injected with the drug, the time clock was started and then the mouse was placed on the hot-plate which was maintained at a constant temperature of between 50 and 55°C.

The time taken by the mouse to stand on its hind-legs and tick its front paws was taken as the end point. The readings of the observed reaction time were done after every 5 minutes for one hour.

ii) Analgesiometer:

With this machine, the pressure used to induce pain on the mouse was recorded. After injecting the mouse with the drug, the reaction pressure was found out by placing the hind-paw of the mouse on the pressing pin and increasing the pressure slowly until the mouse shows signs of pain.

The recordings were done every 5 minutes for a duration of onr hour.

THE DIURETIC ACTIVITY

Six rats were starved for twenty four hours. Each rat was then water-loaded by a stomach tube with 5ml warm water per 100g body weight.

The six rats were then divided into 3 groups of 2 each.

One of the groups was not treated with any drug. This group was the control. For the remaining two groups, one was injected subcutaneously with frusemide and the other with the unknown drug one hour after the water-loading.

After injection of the drugs, the rats were returned to their respective cages for urine collection.

The urine samples were collected under liquid paraffin at time intervals of 35 min., 75min., 135min., 180min., and at 300minutes.

For each urine samples, the volume of urine collected at any given time was read off from the measuring cylinder and the urine flow rate (ml/min) determined.

DETECTION OF METABOLITES IN URINE

In detection of metabolites in urine, the thin layer chromatographic technique was used. The metabolites on the separated according to their solubility in the solvent.

The Rf values, the colour change obtained after spraying with detecting reagents were noted.

For collection of urine samples, 3 rats were water-loaded with 5ml warm water per 100g body weight one hour before administering the drug.

They were then put into one metabolic cage and urine collected before drug administration was the control.

One hour after water-loading, 0.6mg per 100g body weight of the drug was injected subcutaneously and the rats again returned to the metabolic cage for further urine collection.

After collecting the urine, the samples were then run on the TLC against a purt or the parent drug. When the developments of the Chromatoplates was complete, the plates were examined using:-

i) UV light
ii) Exposed to iodine vapour
iii) Sprayed with,
    a) Perchloric acid
    b) P-anesaldehyde.

The spots were marked off with a needle and a copy of the Chromatogramm was made on a sheet of tracing paper.

DETECTION OF THE METABOLITES IN BILE:

Rats of average weight 25 grams was injected intraperitoneally with 0.6ml per 100g body weight urethane to anaesthetist it.

The femoral vein was exposed and separated from the artery and the surrounding tissues using blunt forceps to separate. The portion near the body was clumped with bull-dog clips and cotton threads were passed beneath the vein at both the upper and lower ends of the exposed portion of the vein. The thigh was then tapped gently to fill the vein with blood after which the vein was tied tightly but not too tightly, on the lower end using the thread.

The vein was then lifted using the ends of the threads and carefully nipped at a point midway between the lower and upper portion using a fine small curved pair of scissors.

Care was taken not to cut off the vein. A fine plastic cannular attached to a syringe with the hyperinized salined was pushed carefully into the vein. The clip was removed once the cannular was well inside the vein. The cannular was held firmly using the loose ends of the threads. The cannulation was checked for leakage by pushing some of the saline through the vein.

The vein was used to inject the drug intravenously by replacing the syringe containing the heparinised saline with another syringe containing the drug. Care was taken not to introduce any air bubble.when

When excess bleeding occured, some of the saline was injected to replace the lost blood. After the cannulation of the femoral Vein, the rat was opened at mid-line to expose the bile duct, in the duodenal region of the intestines.

The bile duct was traced to the region just below the bifurcation near the liver. A portion of the bile duct was separated from the tissues and a thread passed beneath the duct at the lower end.

The duct was tied on both ends and cannulation was carried out in the same way as for the femoral vein.

When the cannular was successfully inside the duct, bile started flowing out and this was collected in a sample tube. This was taken to be the control.

The synthetic compound was then injected through the vein and bile collected at a given time intervals.

Injection of the drug intravenously ensured that all the volume of the drug was successfully inside the body.

The collected samples were applied on the chromatoplate using a capillary tube. The applied solution was concentrated on the chromatoplates by a hairdryer.

After the developments was complete, the plates were sprayed the same way as for urine.

The spots were marked off and the chromatogram traced on tracing paper.

RESULTS:

OBSERVATIONS MADE ON WHOLE ANIMAL

TEST	OBSERVATION	INFERENCE
After the intra-peritoneally in-jection of drug, any changes were noted	The corneal, Pedal and righting reflex-did not change.	Drug with no effect on reflexes
Any changes that did occur in the cardio-vascular system after in-jection of the drug were noted.	The heart rate remained the same as before.drug injection.	Drug with no effect on the CVS
Drug effect on the CNS was investigated.	The animal remain-ed normal. No effect on:- ,Muscular inco-ordination ,Drowsiness ,Excitability	Drug probably did not have any effect.

TEST	OBSERVATION	INFERENCE
The drug was injected intraperitoneally and the following observed . Analgesia . Respiratory system effects . Diuretics	There was no change in the breathing rate of the animal. Animal could tolerate some pain after the 15th minute. Animal was producing urine more frequently than the control	The drug probably has no effect on the respiration but may probably have some analgesia and diuretics

INVESTIGATION OF ANALGESIC POTENCY OF FK-∝ USING HOT-PLATE (Temperature 50° to 55°C)

Weight of mouse in grams	DRUG	DOSE	Average reaction time(Secs) before drug Administration	Average reaction time (secs) at 5-minute intervals after drug administration.									
				3 min	5 min	10 min	15 min	20 min	25 min	30 min	35 min	40 min	45 min
35.5	UNICHIN (FK-∝)	0.6mls/ 100g	24.5	24.3	23.7	23	22.7	23.3	23.7	21.3	23	22.3	23.6
26.5			15.7	17.7	14.3	14.7	17.3	15.7	15	15.3	16.7	17	16.3
36.5			20.6	18.7	21	20.3	21.7	21.7	19	21.3	19.7	20.3	22.7
15	SULIDAC	2mg/Kg	13.5	13	13.5	14.5	12.5	14	13.5	12.5	12.5	14	13.0
15			16	15.5	13.5	15.5	15.5	14	15.5	15.5	15	13.5	15.5
25	PETHIDINE	10mg/Kg	21	-	22.5	20	21	19	20.5	22.5	22	21.5	20
15			15	-	15	15	14.5	14.5	14	14	14.5	15.5	13.5

EFFECT OF FK-α ON DIURETIC

DRUG	DOSE	NUMBER OF RATS	URINE OUTPUT (MLS)					Total Vol. (mls)	Total Time (mls (min)	Flow Rate (mls/ min)
			35 min	75 min	135 min	180 min	300 min			
FRUSEMIDE	20mg/kg	2	9	17.5	22.5	23	23.5	95.5	300	0.318
CONTROL	-	2	0.3	3.0	8.0	9.0	9.0	29.3	300	0.098
FK-α	0.6mls/ 100g	2	2	6.5	15m	16	16	55.5	300	0.185

DATA FOR THE MONTH COMPARING THE ANALGESIC POTENCY

i) FE-X

Reaction time before administration = 20.3

Time (minutes)	5	10	15	20	25	30	35	40	45
Average reaction time(sec)	19.67	19.3	20.6	20.1	19.23	18.97	19.8	19.87	20.3

ii) SULIDAC

Reaction time before administration = 14.75

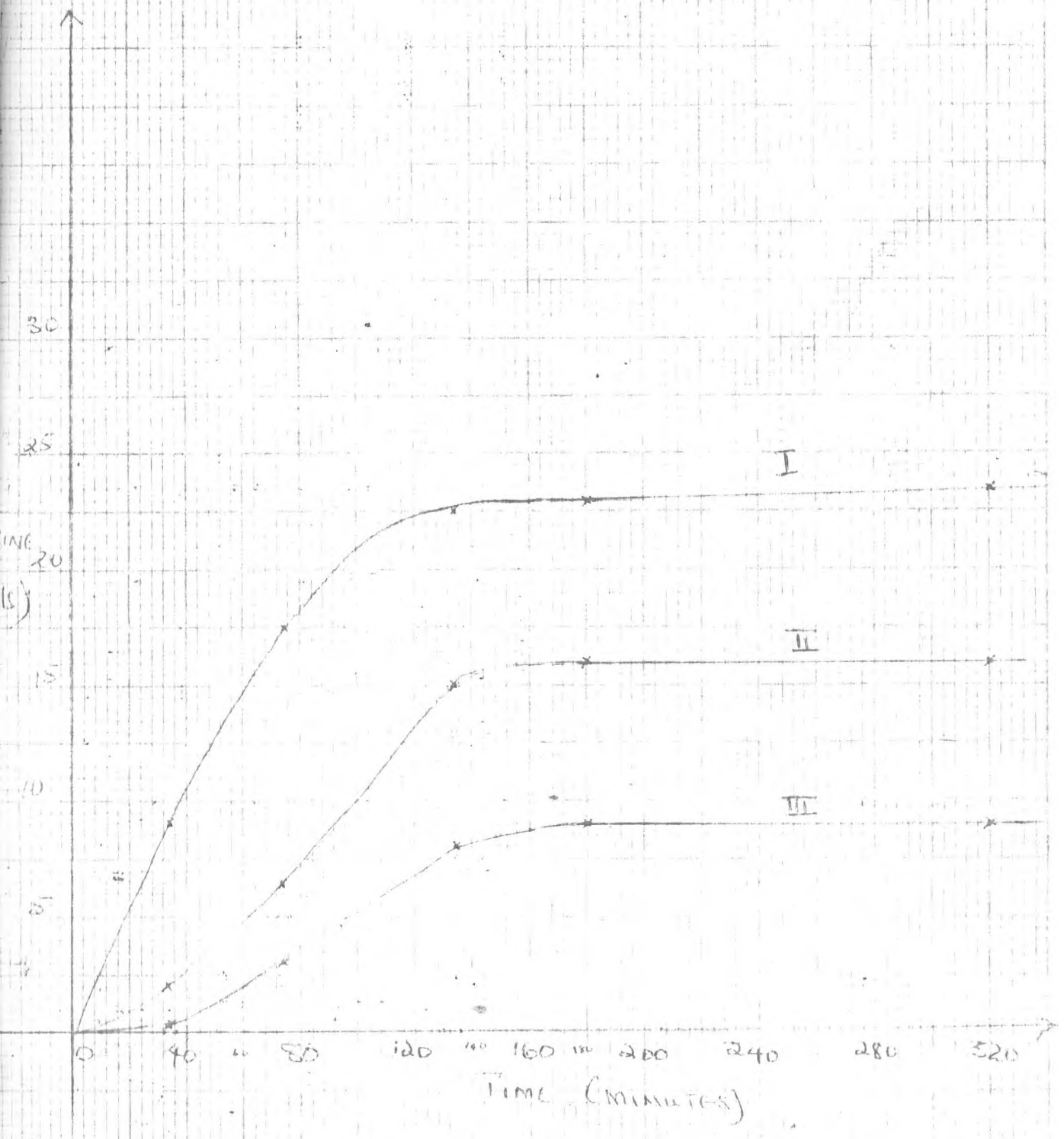
Time (minutes)	5	10	15	20	25	30	35	40	45
Average reaction time(Sec)	13.5	15.0	14.0	14.0	14.5	14.0	13.8	13.8	14.3

ii) PETHIDINE

Reaction time before administration = 18.0

Time (minutes)	5	10	15	20	25	30	35	40	45
Average reaction time(sec)	18.8	17.5	17.8	16.8	17.3	18.3	18.3	18.5	16.8

I: COMPARISON OF THE DIURETIC EFFECT OF FK-A AND FRUSEMIDE.



I - FRUSEMIDE (20 mg/Kg)

II - FK-A (6 c.c/kg of a 400 mg/ml Soln)

III - CONTROL.

DATA FOR GRAPHS COMPARING T.E. DIURETIC ACTIVITY

i) FRUSEMIDE

Time (minutes)	36	75	135	180	300
Volume of urine (mls)	9.0	17.5	22.5	23.0	23.5

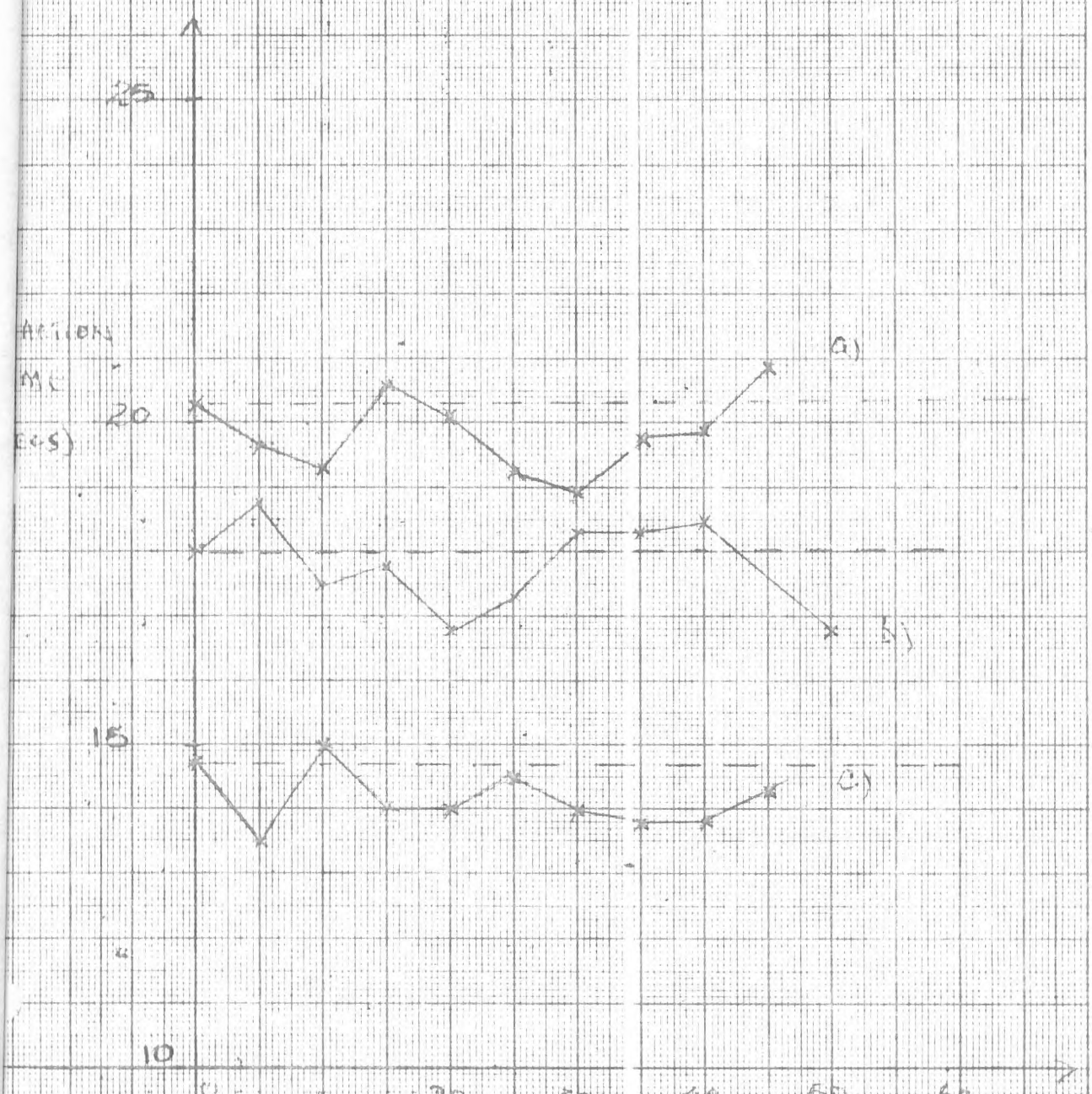
II) FK-α

Time (Minutes)	35	75	135	180	300
Volume of urine(mls)	2.0	6.5	15.0	16.0	16.0

iii) CONTROL

Time (minutes)	35	75	135	180	300
Volume of urine(mls)	0.3	3.0	8.0	9.0	9.0

COMPARISON OF ANALGESIC POTENCY OF FK-A, PETHIDINE AND SULFIDAC



a) — FK-A (0.6 mg/100 ml of 4% mg/ml Sulfin)

b) — Sulfidac (2 mg/kg)

c) — Pethidine (10 mg/kg)

DETECTION OF FK -α IN URINE AND BILE

TEST	OBSERVATION	INFERENCE
The chromatoplates were viewed under the UV light in a chromatoview.	Green spots were observed	The spots indicates the presence of metabolites
The plates were then exposed to Iodine vapour	Brownish yellow spots were observed.	This indicates the presence of the metabolites
The plates were then sprayed with perchloric acid and then heated to 100°C for 5 minutes	Dark-yellow spots were observed.	FK-α metabolites may be present.

RESULTS ON TLC OF FK-506 METABOLITES IN URINE OF RAT

A = URINE COLLECTED BEFORE ADMINISTRATION

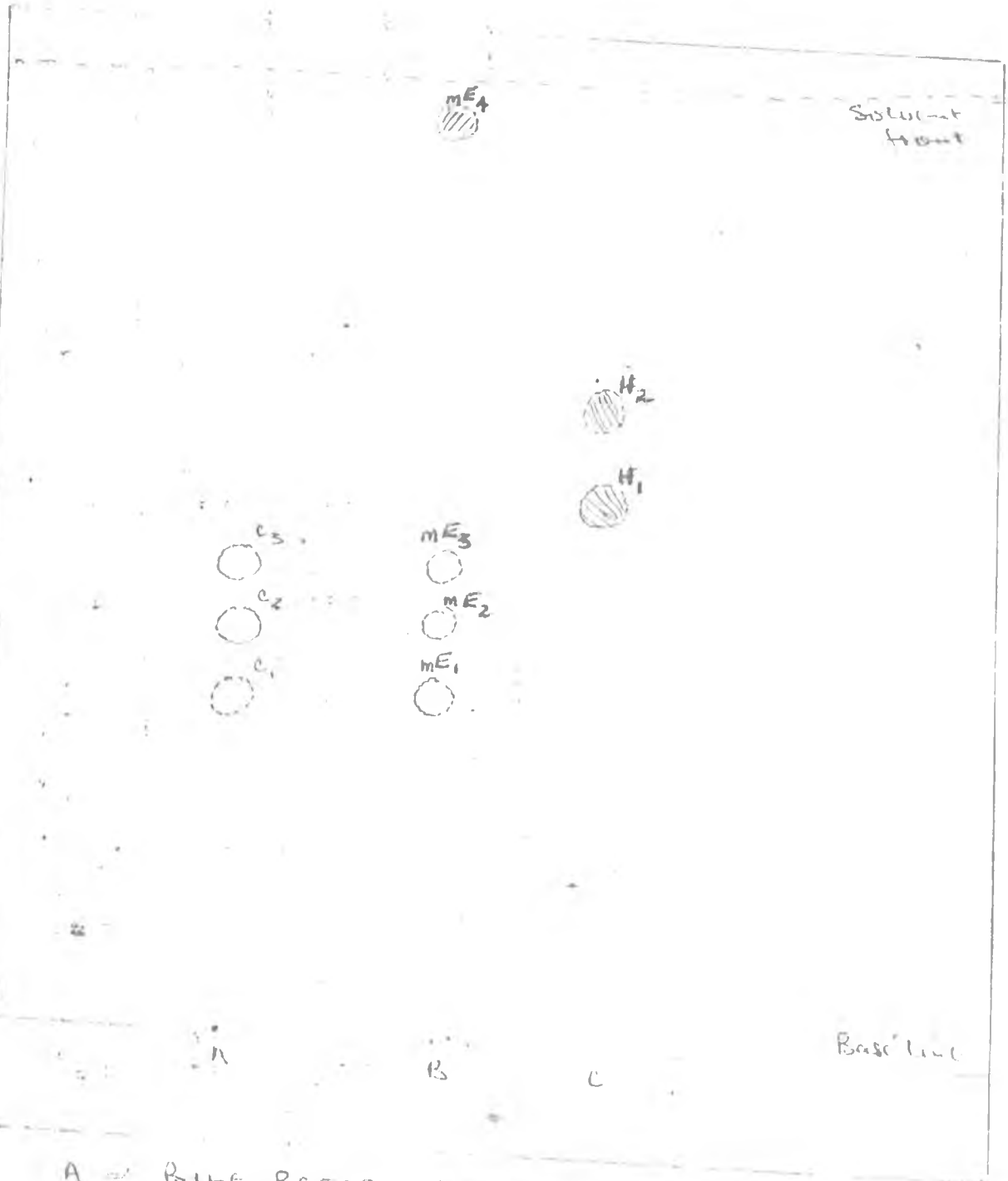
B = METABOLITES IN URINE

C = FK-506 SOLUTION

SOLVENT : BENZENE METHANOL (1:1)

VISUALISATION : UV

RESULTS ON TLC OF FK-A METABOLITES IN BILE OF RAT [30 MINUTES AFTER IV. INJECTION OF DRUG]

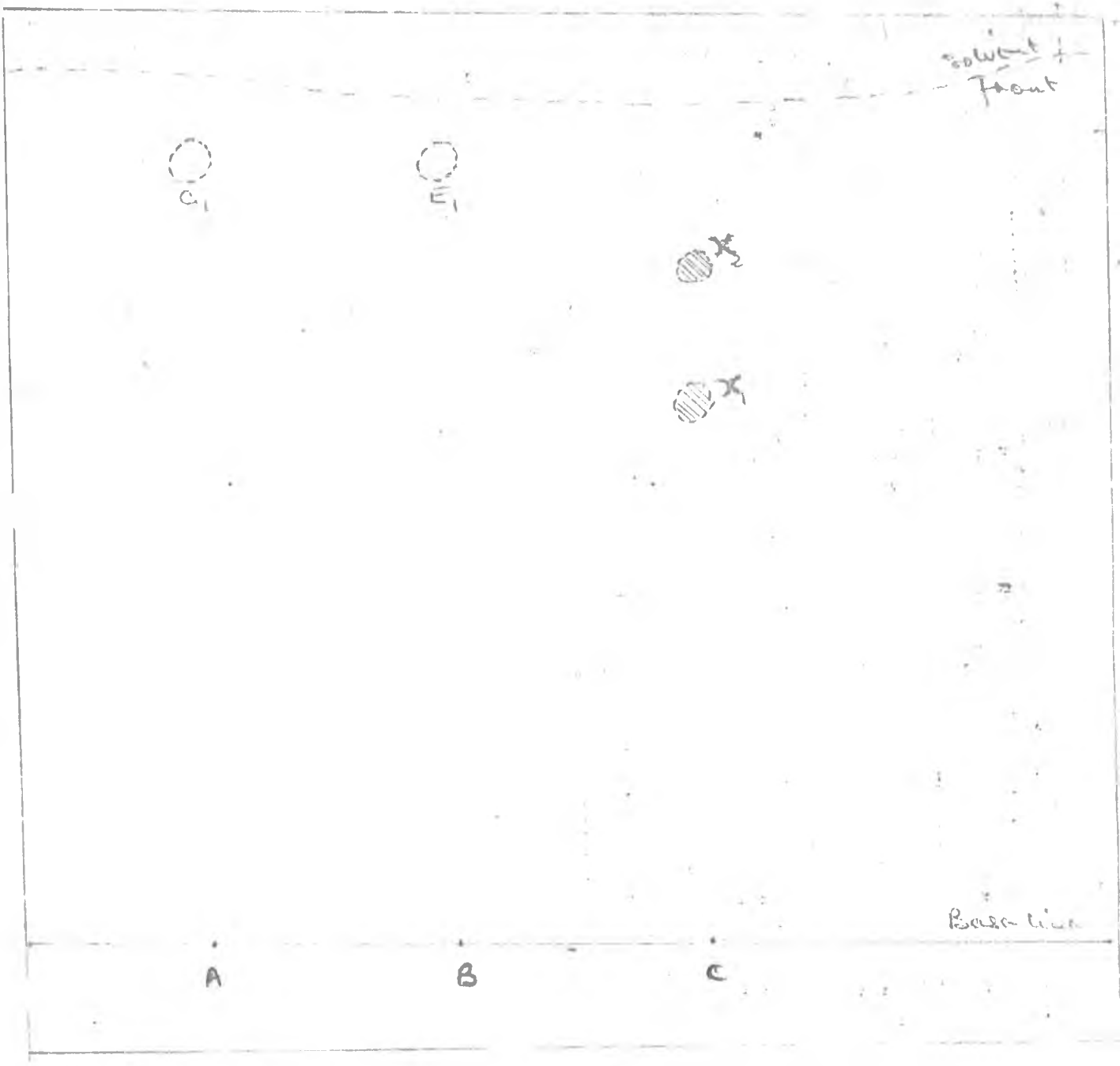


A = BILE BEFORE DRUG ADMINISTRATION
B = METABOLITES IN BILE
C = FK-A SOLUTION

SOLVENT: CHLOROFORM : METHANOL : WATER (65 : 30 . 5)

SPRAYING REAGENT: PERCHLORIC ACID.

RABBIT [30 MINUTES AFTER IV INJECTION]



A = BLOOD BEFORE DRUG WAS INJECTED

B = FK-α IN BLOOD 30 MINUTES AFTER INJECTION

C = FK-α

SOLVENT: BENZENE: METHANOL (1:1)

VISUALIZATION: UV.

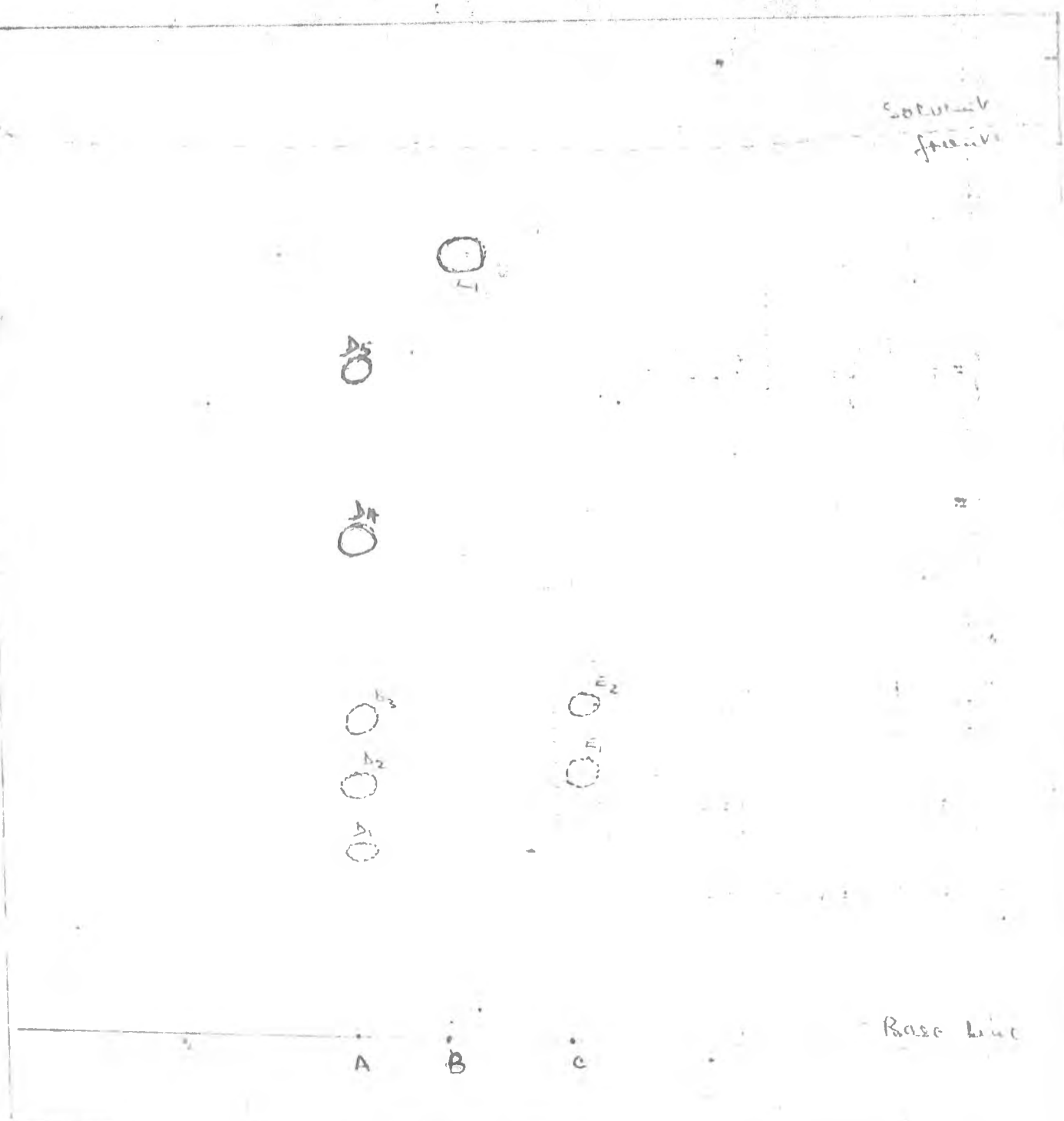
RELATIVE Rf VALUES OF SPOTS OBTAINED IN CHROMATOGRAM FOR URINE AND BILE SAMPLES:

SAMPLE	SPOT		Rf VALUE
FK-α urine samples	FK-α solution	eE_2	0.741
		E_1	0.604
	Metabolites	F_1	0.101
		F_3	0.309
FK-α bile samples	FK-α solution	H_1	0.512
		H_2	0.60
	Metabolites	mE_4	0.824
FK-α blood samples	FK-α solution	x_1	0.634
		x_2	0.796

DETECTION OF DIGOXIN IN BILE

TEST	OBSERVATION	INFERENCE
The chromatoplates were examined under UV light	Brownish spots were observed.	Digoxin may be present in bile
The plates were exposed to iodine vapour.	Brownish yellow spots were observed	This indicates that digoxin metabolites may be present
The chromatoplates were then sprayed with perchloric acid and then heated to 110°C for 5 minutes	Dark yellow spots were observed	Digoxin metabolites may be present
The chromatoplates were sprayed with M-Dinitro Denzene	Greenish spots were observed	Digoxin metabolites may be present

RESULTS ON THIN LAYER CHROMATOGRAM OF DIGOXIN METABOLITES IN BILE OF RAT



A = DIGOXIN METABOLITES IN BILE

B = PURE DIGOXIN

C = BILE ~~COLLECTED~~ COLLECTED BEFORE ADMINISTERING DIGOXIN.

SOLVENT — CHLOROFORM : METHANOL : WATER (65 : 30 : 5)

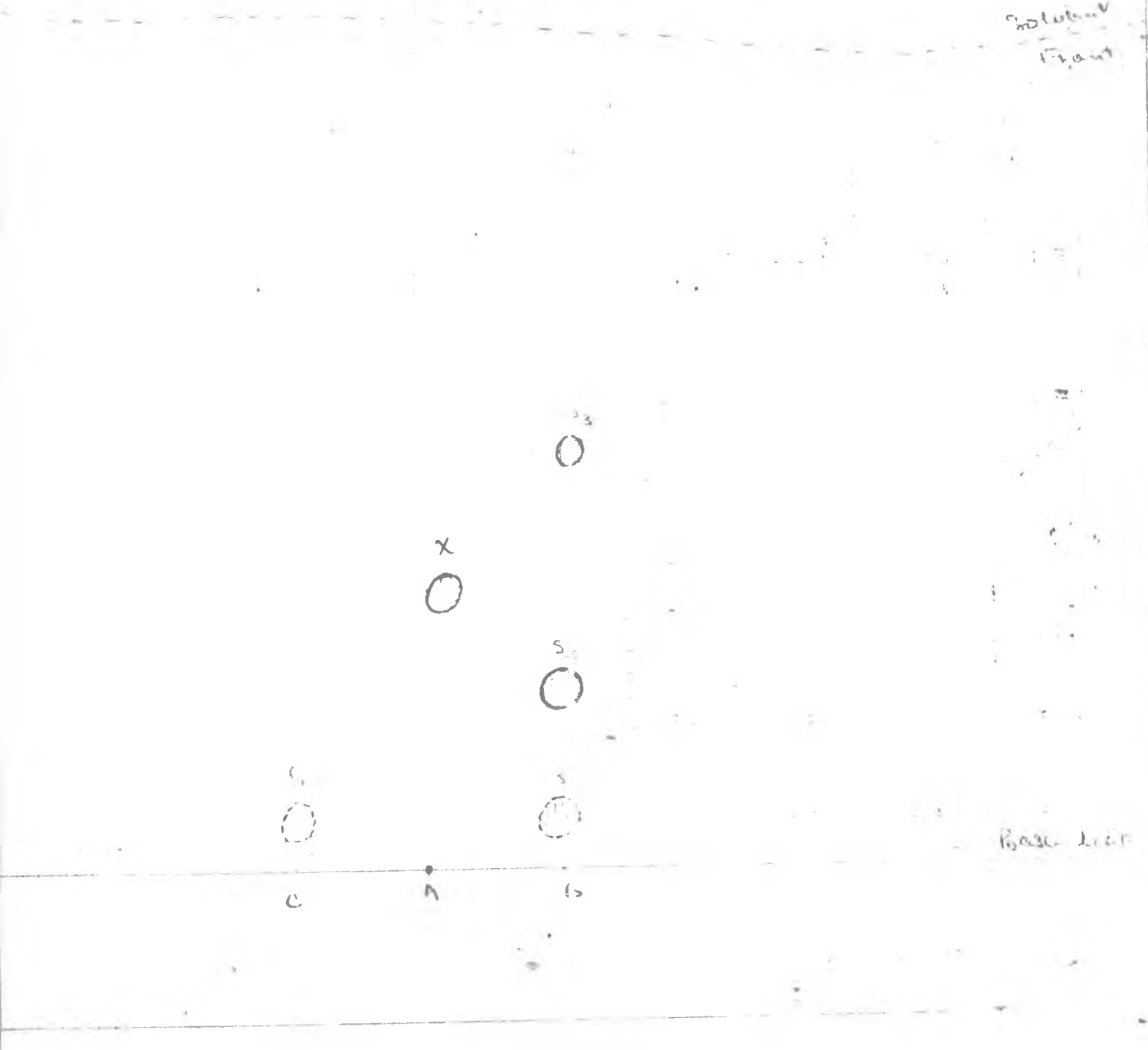
SPRAYING REAGENT — PERCHLORIC ACID

DETECTION OF SULIDAC (redacted) IN URINE OF RAT:

After running the chromatograms, they were subjected to the same detection methods as in detection of the other samples.

TEST	OBSERVATION	INFERENCE
The chromatoplates were viewed under UV light	Brownish spots were observed on the plates.	Sulidac may be present in urine
The plates were then exposed to iodine vapour tank	Brownish-yellow spots were observed.	The metabolites of Sulidac metabolites could be present
The chromatoplates were sprayed with perchloric acid and heated to 110°C for 5 minutes	Dark-yellow spots were observed.	This shows that the metabolites of Sulidac may be present in urine
The chromatoplates were sprayed with M-Dinitro Denzene	Yellowish-green spots were observed	Metabolites of Sulidac may be present in urine

RESULTS ON THIN LAYER CHROMATOGRAM OF SULIBAC METABOLITES IN URINE OF RAT.



A = PURE SULIBAC

C = URINE BEFORE ADMINISTERING SULIBAC

B = SULIBAC METABOLITES IN URINE

SOLVENT : BENZENE : METHANOL : [1:1]

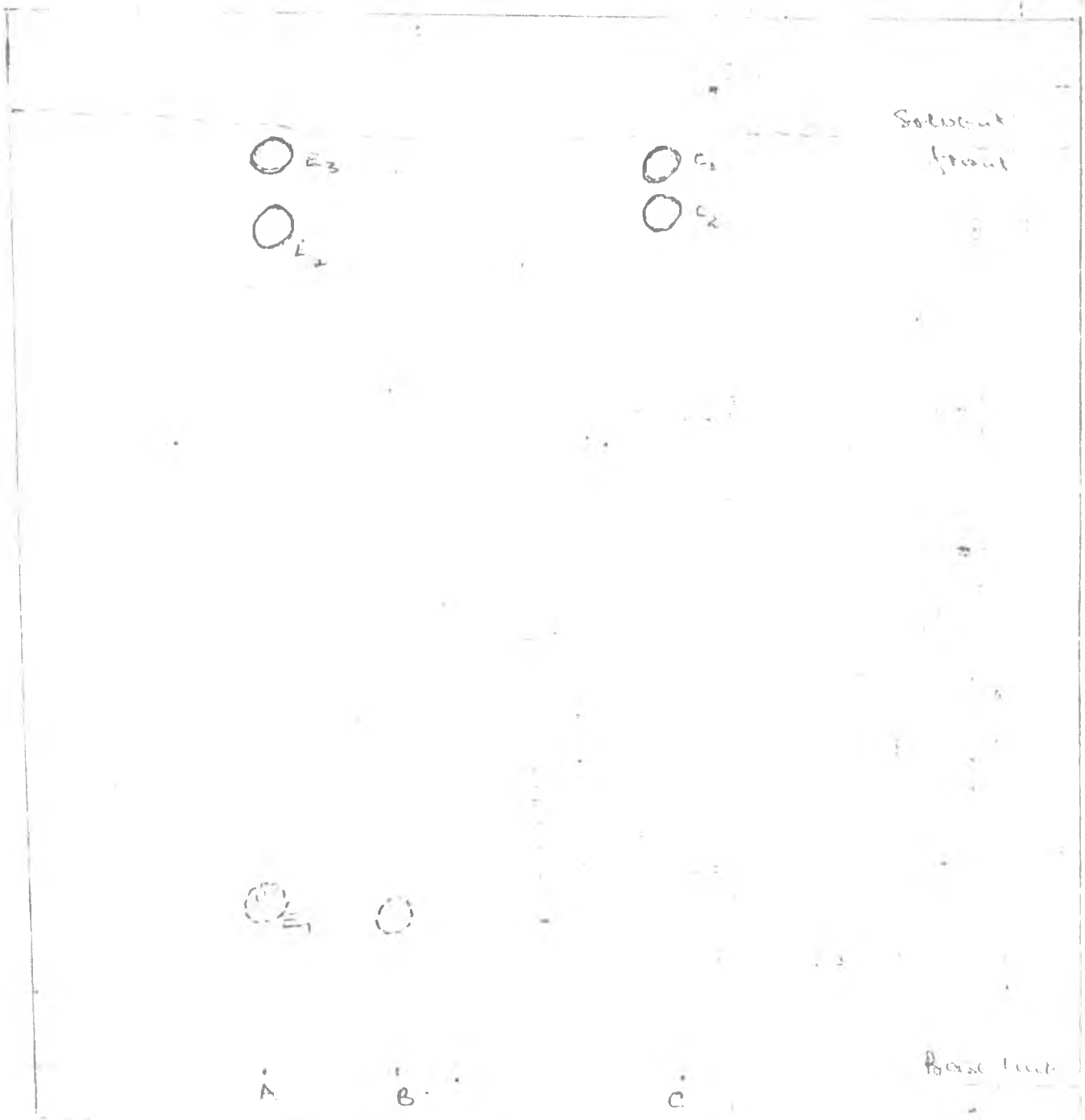
VISUALIZATION : UV

DETECTION OF NUTMEG METABOLITES IN URINE

After running the TLC, the chromatoplates were then viewed under UV light and sprayed with perchloric acid.

TEST	OBSERVATION	INFERENCE
The chromatoplates were examined under UV light	Light greenish spots were observed	Nutmeg metabolites may be present in urine.
The chromatoplates were sprayed with perchloric acid	Brownish spots were observed for both the sample and the pure nutmeg	Nutmeg Metabolites may not be present in urine since all the spots corresponded with the spots of the Nutmeg residue
The chromatoplates were sprayed with M-Dinitro Benzene	Greenish spots were observed for both the test sample and pure nutmeg	Since the spots of the test sample and pure Nutmeg were equivalent, then no metabolites were present in urine

METABOLITES IN URINE OF RAT [ONE HOUR AFTER ADMINISTRATION]



A = NUTMEG IN URINE

B = URINE BEFORE ADMINISTRATION

C = NUTMEG SUSPENSION.

SOLVENT : CHLOROFORM : METHANOL : WATER (65 : 30 : 5)

SPRAYING REAGENT : PERCHLORIC ACID.

DISCUSSION

From the result of the present work, it was shown that the synthetic compound, FK-9, had no effect on the cardio-vascular system, respiratory system, and central nervous system. It did not abolish any of the reflexes even after a duration of one hour.

When the compound was tested for any analgesics and diuretic activity, it was found to have some significant effect on analgesia and diuretic.

In the sudy of analgesia, Pethidine and Sulidac were used as standard drugs. It should be pointed that when studying the biological activities of a compound in different groups of animals result obtained may vary from theoretical ones due to factors like, sex, pathological conditions of the animal, metabolic rate and even the body temperature.

On screening for the metabolites, it was observed that they were present in both bile and urine.

In urine, two spots representing the metabolites were observed. They had Rf values of 0.101 and 0.309 while the Rf values of the synthetic compound before it was injected were 0.74 and 0.604. The Rf values shows that these spots represented different metabolic products.

In bile, only one spot was observed. It had an Rf value of 0.824 while the Rf values of the synthetic compound before it was administered were 0.512 and 0.60. When blood obtained after intravenous injection of the compound was run on the chromatogram, no spots was obtained but two spots of Rf values 0.634 and 0.798 were obtained when the compound was run on the chromatogram after before adminidtration. This showed that the drug was not biotransformed in blood.

The chromatograms of the standardizing drugs showed that:-

a) Digoxin was biotransformed in bile and this was showed by the two spots obtained on the chromatogram.

b) Sulidac after biotransformation was excreted through urine. Two spots, representing the metabolites were obtained.

c) Mutamycin suspension which was given orally had two spots

when the urine sample was run on the chromatogram. These spots corresponded with the two spots obtained after the suspension was run on the chromatogram before it was administered.

These showed that although Nutmeg is excreted through urine it is excreted in unchanged form.

CONCLUSION

From the results and the above discussion, it can be concluded that the synthetic compound FK-X have some analgesic and diuretic activities.

After biotransformation of the compounds, the metabolites are excreted through bile and urine and biotransformation of the compound does not occur in blood.

Though other biological activities of the compound were invetigated, none was found to be positive.

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