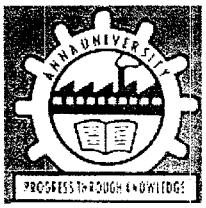

ANALYSIS OF GASTRIC ASPIRATES IN POISONING BY USING THIN LAYER CHROMATOGRAPHY

A PROJECT REPORT

Submitted by

LAKSHMI .S

in partial fulfillment for the award of the degree

of

BACHELOR OF TECHNOLOGY

in

BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY

ANNA UNIVERSITY: CHENNAI 600 025

APRIL 2010

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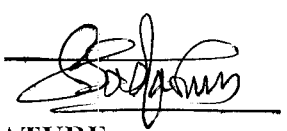
BONAFIDE CERTIFICATE

Certified that this project report **"Analysis of Gastric Aspirates in poisoning by using Thin Layer Chromatography"** is a bona fide work of **LAKSHMI S** who carried out the project work under my supervision.



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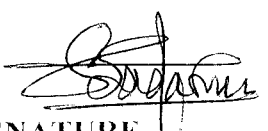

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The report of the project work submitted by the above student in partial fulfillment for the award of Bachelor of Technology degree in Biotechnology of Anna University was evaluated and confirmed to be a report submitted by the above student. It was submitted for the viva voce examination on 20.04.2010.

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CERTIFICATE

This is to certify that the dissertation work entitled **"Analysis of gastric aspirates in poisoning by using Thin Layer Chromatography"** is a bonafide record of the work carried out by **Ms. Lakshmi. S** at **Madras Medical College, Chennai,** in partial fulfillment for the degree of **B. Tech in Biotechnology** for **Kumaraguru College of Technology, Coimbatore**. The work embodied in this dissertation had been done by the candidate herself under my guidance and supervision during **January 2010 to March 2010.** This has not previously formed the basis for the award of any degree, diploma, fellowship or any other similar title.

GUIDE

(Dr. K.Ramadevi)

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LAKSHMI S

ABBREVIATIONS

TLC	-	Thin Layer Chromatography
OP	-	Organo Phosphorous / Organaophosphates
AChE	-	Acetyl Choline Esterase
NTE	-	Neuropathy Target Esterases
UV	-	Ultra Violet
NaOH	-	Sodium Hydroxide
DPC	-	Diphenylcarbazone

ABSTRACT

This project deals with the qualitative analysis of organophosphates, salicylates and barbiturates in gastric aspirates of patients by using Thin Layer Chromatography where in the stomach contents of the patients affected by the above mentioned substances are analyzed.

Organophosphates, Organochlorides and Carbamates form the three main classes of pesticides. Organochlorides mainly operate by disrupting the sodium / potassium balance of the nerve fiber, forcing the neurons to transmit the signal continuously. On the other hand Organophosphates and carbamates act by inhibiting the enzyme Acetylcholinesterase. This results in the uncontrolled activity of Acetylcholine, a chemical neuro-transmitter, which is involved in the transmission of nerve signals. Thus an activated acetyl choline transmits nerve impulse indefinitely causing a variety of symptoms such as weakness or paralysis. Here pesticides of the class organophosphates were analyzed.

Over dosage of drugs are found to produce toxicity to the biological system. Affected individuals show symptoms like weakness, drowsiness, sometimes it might turn out to be fatal.When a patient was suspected to have consumed high dosage of any drug like barbiturates or salicylates the patient's stomach contents were analyzed Thin Layer Chromatography. The maximum absorbance for barbiturates samples were also determined to study the absorbance of the barbiturates and these values were compared with the normal samples devoid of any drug.

Patients ingested with poison or patients who have consumed poison mostly arrive at the hospital at a semi-conscious or unconscious state. Hence it is very important that they should be treated at the earliest. Thin layer chromatography allows us to analyze the sample within a very short period of time. Moreover it can be carried out on a small scale because it is a very simple technique and it is cost-effective.

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INTRODUCTION

1. INTRODUCTION

A toxin or a poison is any chemical that is capable of producing detrimental action on a living organism. Poisoning is the harmful effect that occurs when a toxic substance is swallowed, is inhaled, or comes in contact with the skin, eyes, or mucous membranes, such as those of the mouth or nose. As a result of damage caused due to these agents, there is an alteration of structural components or functional processes which may be injurious to health or may even be fatal.

It is assumed that signs and symptoms of poisoning start the moment exposure to toxins occur. Although this is true in some cases, it is an incorrect assumption for many toxic exposures. In some instances, the onset of toxic effects is not immediately observed. It might take hours or days for the toxins to manifest themselves in the form of symptoms, when consumed by the patient. Certain pesticides, heavy metals, and timed-release dosage forms of pharmaceuticals illustrate a delayed onset. Sometimes, people who are exposed to a wide variety of toxic substances each day, do not often display any symptoms of toxicity.

Poisoning is the most common cause of nonfatal accidents in the home. Young children are particularly vulnerable to accidental poisoning at home, as are older people, often from confusion about their drugs. Poisoning may also be a deliberate attempt to commit murder or suicide. The damage caused by poisoning depends on the poison, the amount taken, and the age and underlying health of the person who takes it. Some poisons are not very potent and cause problems only with prolonged exposure or repeated ingestion of large amounts. Other poisons are so potent that just a drop on the skin can cause severe damage. Some poisons cause symptoms within seconds, whereas others cause symptoms only after hours or even days. Some poisons cause few obvious symptoms until they have damaged vital organs such as the kidneys or liver, sometimes permanently.

Poisoning maybe classified into different types as follows.

- Acetaminophen poisoning
- Pesticide poisoning
- Caustic substances poisoning
- Hydrocarbon poisoning
- Carbon monoxide poisoning
- Aspirin poisoning
- Iron poisoning
- Lead poisoning
- Barbiturates poisoning

Pesticide poisoning especially organophosphate pesticide poisoning is one of the most common forms of poisoning. Most serious insecticide poisonings result from the organophosphate and carbamate types of insecticides, particularly when used in suicide attempts. Examples of organophosphates include Malathion, parathion, diazinon, dichlorvos, chlorpyrifos, and sarin. Some of these compounds are derived from nerve gases. Pyrethrins and parathyroid, which are other commonly used insecticides, are derived from flowers and usually are not poisonous to humans. The most common exposure scenarios for pesticide-poisoning cases are accidental or suicidal poisonings, occupational exposure, by-stander exposure to off-target drift, and the general public who are exposed through environmental contamination.

Amongst drug poisoning barbiturates, acetaminophen, aspirin and other salicylates are more frequent because drugs like aspirin are commonly available in the medical shops. All drugs, especially in large doses or when taken over long periods of time, can initiate a toxic condition. Alcohol and barbiturates, together, result in an intensified alteration of physiological state that is frequently dangerous

These drugs affect the nervous system and often cause adverse reactions in high concentrations. Alcohol and other nervous system depressants, such as barbiturates and narcotics, taken in sufficiently large doses, can result in coma and convulsions.

Excessively high doses of stimulants such as amphetamines result in blurred vision, spasms, heart irregularities, and respiratory failure. In addition, continued use of both stimulants and depressants can lead to addiction and tolerance for toxic doses. Over dosage of an analgesic like aspirin can result in acid-base disturbances, spontaneous bleeding, and convulsions. Aspirin poisoning is the most common among salicylate poisoning and acute aspirin poisoning leads to death of the individual.

Several patients who are exposed or ingested with poisons arrive at the hospital. They arrive at the hospital mostly after the onset of the symptoms. Some may have consciousness while some may not have. The toxicity produced in patients depends upon the poison that has affected them. Irrespective of the condition of the patient, it becomes necessary to diagnose the patients and provide them treatment soon after they arrive at the hospital.

Analytical toxicology is concerned with the detection, identification and measurement of drugs and other foreign compounds (xenobiotics) and their metabolites in biological and related specimens.

In particular, ultraviolet (UV) and infrared (IR) spectrophotometry, together with visible spectrophotometry (colorimetry), and paper and ion-exchange column chromatography are the widely used techniques that are employed to detect the toxins. More recently, paper chromatography has been largely superseded by thin-layer chromatography (TLC) as this latter technique offers advantages of speed of analysis and lower detection limits.

This project deals with the analysis of the gastric aspirates of the patients and finding out the presence/ absence of the pesticide poison organophosphates and also the presence/ absence of over dosage of the drugs , barbiturates and salicylates, by using Thin Layer Chromatography technique . It also deals with determining the maximum absorbance of barbiturates and comparing it with the normal patients' sample.

The objectives of this project are listed as follows.

- Collecting patients' gastric aspirate samples
- Extracting the specific drug or pesticide poison from the sample
- Performing TLC with the extract by running the extract along with specific standards.

- Comparing a normal patients' chromatogram with the chromatogram of the affected patients.
- Determining the maximum absorbance of the patients' samples that tested positive for barbiturates and comparing it with that of normal patients' sample by using UV- Spectrophotometry.

REVIEW OF LITERATURE

REVIEW OF LITERATURE:

2.1 Pesticide toxicity:

2.1. A) The effect on global mortality:

Evidence is accumulating that pesticide self-poisoning is one of the most commonly used methods of suicide worldwide, but the magnitude of the problem and the global distribution of these deaths is unknown. Literature has been systematically reviewed to estimate the number of pesticide suicide in each of the World Health Organization's six regions and the global burden of fatal self- poisoning with pesticides. Various data sources like Medline, EMBASE, etc were used and the proportion of the world's suicides due to pesticide poisoning was estimated. (David et al,)

It was estimated that there are 258,234 (plausible range 233,997 to 325,907) deaths from pesticide self-poisoning worldwide each year, accounting for 30% (range 27% to 37%) of suicides globally and was concluded that self- poisoning accounts for about one-third of the world's suicides. Recent extrapolations of data from a few countries in Asia suggest that there may be 300,000 suicides by deliberate ingestion of pesticides annually (Buckley et al, 2006)

2.1. B) Organophosphate toxicity:

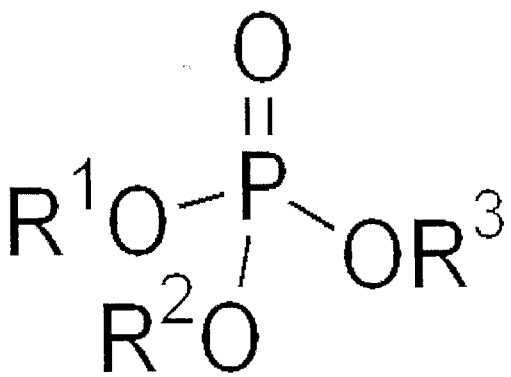
It was found that OP insecticides appear to be the most commonly ingested pesticides in rural Asia, accounting for around two thirds of cases.(Srinivas et al. 2005) Organophosphorus pesticide self-poisoning is an important clinical problem in rural regions of the developing world, and kills an estimated 200 000 people every year. Of the estimated 500 000 deaths from self-harm in the region each year, about 60% are due to pesticide poisoning.(Lotti et al. 2001) In India, the state of Andhra Pradesh, southern India, is an area of intensive agricultural production. Pesticide use is high, and the state has one of the highest reported rates of pesticide poisoning in India. (Srinivas et al. 2005)

Many studies estimate that OP pesticides are responsible for around two-thirds of these deaths—a total of 200 000 a year. Deaths from unintentional OP poisoning are less common than those from intentional poisoning and seem to be more common in regions

where highly toxic OP pesticides are available. In a large cohort of Sri Lankan patients poisoned with WHO Class II OP pesticides, no deaths resulted from unintentional poisoning. (Eddleston et al, 2008)

Mode of action of organophosphate pesticides:

FIG 2.1 Structure of OP Molecule



Organophosphates are those groups of compounds that have the potential to irreversibly inhibit choline esterases including acetyl choline estrase (AChE) and neuropathy target esterases (NTE), which was previously known as neuro toxic esterase. Most compounds have been designed to sere as insecticides from their ablity to inhibit AChE irreversibly .Serious human exposure to these compounds result in cholinergic central nervous system findings. muscle weakness as a result of AChE inhibition. The organophosphorous compounds are irreversible inhibitors of AChE because the resultant phosphorylated enzyme is highly stable.(Yurumez et al. 2007)

The enzyme AChE hydrolyses the Ach once Ach is released from the presynaptic terminal of a synapse. These OP compounds mimic the structure of Ach. Hence they are structurally complementary to the target enzymes that hydrolyze Ach (choline esters). This results in altered nerve signaling. (Moretto , 1998)

The OP compounds that produce delayed neurotoxicity, phosphorylate specific esterase in the nervous tissue. This enzyme which was formerly termed as neurotoxic

esterase has been renamed as neuropathy target esterase. It is found in spleen, nervous system, muscle and blood cells. In humans, the lymphocyte NTE levels are a reliable indicator of enzyme inhibition in the nervous system.

When a non-neurotoxic organophosphorous compound is administered, AChE and non- specific cholinesterase are inhibited in the brain; approximately 6% of the esterases remain active and can be selectively inhibited by OPs producing delayed neurotoxicity. OP compounds act in more than one way once they enter the biological system. (Peter et al, 2006)

2.2) Drug toxicity:

2.2. A) Barbiturates toxicity:

Between the 1920s and the mid-1950s, practically the only drugs used as sedatives and hypnotics were barbiturates.(Gary et al, 1983) From a chemical point of view, these drugs are closed-chain ureic compounds, whose nucleus is malonylurea (a combination of urea, a product present in animal excrement, and malonic acid, an acid derivative taken from apples). Barbiturates were synthesized in 1864 by Adolf von Baeyer, though the synthetic process was developed and perfected by the French chemist Edouard Grimaux in 1879, making possible the subsequent widespread development of barbiturate derivatives

The first of the barbiturates to come onto the market was diethyl-barbituric acid, also known as barbital, malonal, or gardenal. By means of small modifications to the chemical structure of the barbituric acid molecule, more than 2500 different agents were synthesized. One of them, perhaps that most widely used subsequently was Phenobarbital. Phenobarbital was employed in therapy as a hypnotic. Several new barbiturates were synthesized in the later years and these compounds were used as hypnotic, antiepileptic drugs, in "sleep cure" and in intravenous anesthesia. (Lowinson et al, 1997)

Chemists from different universities and pharmaceutical companies managed to synthesize over 2500 barbiturate derivates. The differential pharmacokinetic properties of

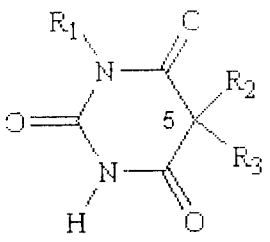
these agents made it possible to draw up a practical clinical classification, based on the duration of their pharmacological action. Thus, the barbiturates in the category of short or intermediate action (secobarbital, amobarbital, pentobarbital) were employed initially as hypnotics, whilst those of prolonged action (phenobarbital) were widely used as anxiolytics and anticonvulsants; ultrashort-acting agents, notably sodium thiopental, were especially useful as anesthetic inducers for minor operations.(Goldfrank, 1994)

Despite these therapeutic uses, barbiturates became less popular due to the adverse effects caused by them in the form of overdosage. Doses 4 6 times higher than the therapeutic dose as hypnotics of the short-acting barbiturates (400 600 mg/day of amobarbital, secobarbital, or pentobarbital) brought about, if the treatment was sufficiently prolonged, authentic withdrawal syndromes when use was stopped. In relation to the frequent cases of death by overdose, given the small therapeutic margin of these substances, it should be pointed out that this was a common method in suicide attempts. The lethal effect of these compounds was such that a mixture of barbiturates with other substances was even employed in some USA states for the execution of prisoners sentenced to death.(Bronstein et al, 2008)

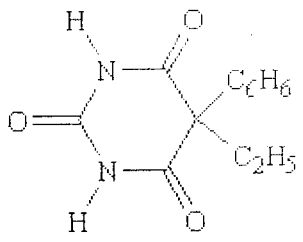
Due to the lethal effects of these compounds, measures were taken restricting the prescription of prolonged acting sedative barbiturates especially during the 1970s. However, the use of barbiturates is circumscribed to quite specific therapeutic applications. Thus, phenobarbital and butabarbital are still used as sedatives in cases of gastrointestinal and asthmatic functional disorders, as well as to antagonize the adverse central stimulant effects of some drugs, such as ephedrine, dextroamphetamine, or theophylline (Nishiyama et al, 2002). Phenobarbital is also used in cases of withdrawal syndromes of hypnosedative agents. In the field of neurology, barbiturates (phenobarbital and primidone) are still employed, not only in the treatment of certain types of epilepsy (partial and tonic-clonic generalized seizures), but also in the emergency treatment of some types of convulsions, such as those associated with tetanus, eclampsia, cerebral hemorrhage, status epilepticus, or different forms of poisoning. As intravenous anesthetic inducers, ultrashort-acting barbiturates are of use, mainly thiopental and methohexital, the latter also being administered rectally in children or as a sedative in some diagnostic imaging explorations. (Francisco lopez munoz et al, 2005)

Mode of action of barbiturates:

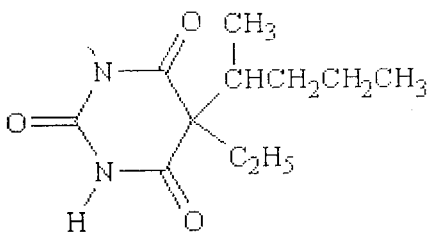
FIG 2.2 Structure of Barbiturates



general structure



phenobarbital



pentobarbotal

Barbiturates bind to specific sites on gamma-aminobutyric acid (GABA)-sensitive ion channels found in the central nervous system (CNS), where they allow an influx of chloride into cell membranes and, subsequently, hyperpolarize the postsynaptic neuron.

GABA and glycine are the major inhibitory neurotransmitters in the CNS. Barbiturates enhance GABA-mediated chloride currents by binding to the GABA-A receptor-ionophore complex and increasing the duration of ionophore opening. This potentiates and prolongs the inhibitory actions of GABA. At high doses, barbiturates stimulate GABA-A receptors directly in the absence of GABA. Barbiturates also block glutamate (excitatory neurotransmitter) receptors in the CNS. Barbiturates affects the central nervous system, induces respiratory depression, cardiovascular depression, etc.(Hadden et al, 1969)

Central nervous system effects

Barbiturates mainly act in the CNS, though they may indirectly affect other organ systems. Direct effects include sedation and hypnosis at lower dosages. The CNS depressant effect mimics that of ethanol. The lipophilic barbiturates, such as thiopental, cause rapid anesthesia because of their tendency to penetrate brain tissue quickly. Barbiturates all have anticonvulsant activity because they hyperpolarize cell membranes. Therefore, they are effective adjuncts in the treatment of epilepsy.

Pulmonary effects

Barbiturates can cause a depression of the medullary respiratory center and induce respiratory depression. Patients with underlying chronic obstructive pulmonary disease (COPD) are more susceptible to these effects, even at doses that would be considered therapeutic in healthy individuals. Fatality from barbiturate overdose is usually secondary to respiratory depression and subsequent pneumonia.

Cardiovascular effects

Cardiovascular depression may occur following depression of the medullary vasomotor centers; patients with underlying congestive heart failure (CHF) are more susceptible to these effects. At higher doses, cardiac contractility and vascular tone are compromised, which may cause cardiovascular collapse. (Keith et al)

2.2. B) Salicylate toxicity:

Salicylic acid (the compound that can be obtained from the bark of a willow tree) is a beta hydroxy acid. This colorless crystalline organic acid is widely used in organic synthesis and functions as a plant hormone. It is derived from the metabolism of salicin.

In addition to being a compound that is chemically similar to but not identical to the active component of aspirin (*acetylsalicylic acid*), it is probably best known for its use in anti-acne treatments. The salts and esters of salicylic acid are known as salicylates.

These molecules have been shown to possess phyto- and chemotherapeutic activities as analgesic drugs. In recent decades, aspirin has become the focus of extensive investigation into anti-proliferative and anticancer activities. (De Wolff, 1994)

Over dosage of salicylates particularly over dosage of aspirin is proven to cause lethal effects. Accidental ingestion of large quantities of salicylates causes death. (Brenner et al, 1982)

The toxic effects of salicylates are complex. Respiratory centers are directly stimulated. Salicylates cause an inhibition of the citric acid cycle and an uncoupling of oxidative phosphorylation. In addition, lipid metabolism is stimulated, while amino acid metabolism is inhibited. Catabolism occurs secondary to the inhibition of ATP-dependent reactions with the following results:

- Increased oxygen consumption
- Increased carbon dioxide production
- Accelerated activity of the glycolytic and lipolytic pathways
- Depletion of hepatic glycogen
- Hyperpyrexia

(Chapman et al, 1989)

Acid-base disturbances vary with age and severity of the intoxication. Initially, a respiratory alkalosis develops secondary to direct stimulation of the respiratory centers. This may be the only consequence of mild salicylism. The kidneys excrete potassium, sodium, and bicarbonate, resulting in alkaline urine.(Danel, 1988)

Mortality/Morbidity

A 16% morbidity rate and a 1% mortality rate are associated with patients presenting with an acute overdose. The incidence of morbidity and mortality of a patient with chronic intoxication is 30% and 25%, respectively.

- The following 4 categories are helpful for assessing the potential severity and morbidity of an acute, single event, nonenteric-coated, salicylate ingestion:
    - Less than 150 mg/kg - Spectrum ranges from no toxicity to mild toxicity
    - From 150-300 mg/kg - Mild-to-moderate toxicity
    - From 301-500 mg/kg - Serious toxicity
    - Greater than 500 mg/kg - Potentially lethal toxicity

Data from the American Association of Poison Control Centers' annual report indicate that, in 1998, a total of 14,253 exposures to salicylates were reported; of which, 3837 exposures were in patients younger that 6 years, and 5053 exposures were in patients older than 19 years. Of the total exposures for that year, 33 deaths were reported. These numbers include only pure aspirin formulations; toxic exposures to pharmaceuticals with aspirin in combination with other drugs are not included in this report (Keith et al)

Mode of action of Salicylates:

Salicylates impair cellular respiration by uncoupling oxidative phosphorylation. They stimulate respiratory centers in the medulla, causing primary respiratory alkalosis, which is often unrecognized in young children. Salicylates simultaneously and independently cause primary metabolic acidosis. Eventually, as salicylates disappear from the blood, enter the cells, and poison mitochondria, metabolic acidosis becomes the primary acid-base abnormality. (Done et al. 1971)

Salicylate poisoning also causes ketosis, fever, and, even when systemic hypoglycemia is absent, low brain glucose levels, increased renal Na, K and water loss results but imperceptible respiratory water loss due to hyperventilation leading to dehydration.(Gilman , 1980)

Salicylates are weak acids that cross cell membranes relatively easily; thus, they are more toxic when blood pH is low. Dehydration, hyperthermia, and chronic ingestion increase salicylate toxicity because they result in greater distribution of salicylate to tissues. Excretion of salicylates increases when urine pH increases.(Danel , 1988)

Causes

Onset of chronic salicylism may be insidious; elderly individuals may consume an increasing amount over several days to alleviate arthralgias, subsequently becoming confused because salicylate pharmacokinetics changes at higher concentrations. This may lead to a perpetual spiral of increased salicylate consumption and increased confusion. Similar scenarios occur in persons with underlying psychiatric disorders. (Gilman, 1980)

2.3) Methods of analyzing poison:

Various chromatographic methods are employed in the analysis of toxins. Toxins from shell fish have been isolated and detected using thin layer chromatography technique, gas chromatography, liquid chromatography. Mycotoxins were analysed using TLC and the results that were obtained were found to be reliable. (Egmond et al. 2004).

Several healthcare industries used TLC for screening various drugs. The principle of TLC is very simple. TLC system for clinical use is a device intended to separate one or more toxins or drugs or compounds from a mixture. The mixture of compounds is absorbed onto a stationary phase or thin layer of inert material e.g., cellulose, alumina, etc.) and eluted off by a moving solvent (moving phase) until equilibrium is achieved between the two phases. The moving solvent rises as a result of capillary action on the plate and once it has reaches ¾th of the plate, it is taken out from the solvent chamber and the results are visualized. Different spraying agents are used for visualizing the results depending on the analyte that has to be separated. Spraying agents helps us to visualize the results with the naked eye. Otherwise the results are viewed under ultraviolet radiation and they are documented. (Hummel D.O, 2000)

The factors involved and chemical requirements for color are mentioned below.

1. Behavior in organic solvents

2. Silica Gel polarity and pH

3. Functional groups responsible for interaction with the reagent

4. pH and chemicals of the color developer

5. Mechanism of reaction

(Horowitz, 2000)

The Thin Layer Chromatography technique is used to compounds that are present in tablets containing simple or over the counter analgesic medications.(Moffat et al. 1986). It has also been used for analyzing several compounds from bulk drug and tablet formulations by using appropriate solvent system. (Hummel D.O, 2000)

Urine drug screening is also carried out by TLC wherein the stationary phase is usually an absorbent like silica gel, which is spread on a solid support. Concentrated drug sample extracts and drug standards are applied as aeries of spots along the bottom of the plate and allowed to dry. (William, 1991). The plate is then placed in a closed tank in which the absorbent layer makes contact with the developing solvent below the applied spots. The solvent moves up the plate by capillary action, dissolving and separating the components of the extracts. When the solvent has reached a predefined distance, the plate is taken out of the chamber and the solvent is evaporated from the plate by means of drying. Each individual drug migrates to a certain distance. The presence of the drug is visualized by spraying the plate with suitable spraying agents. Several sprays may be used in sequence to aid in identification of the compounds.This procedure is similar to the general chromatography technique apart from the steps involved in preparing the urine extract.(Hummel D.O, 2000)

It has also been studied that spots on the TLC chromatogram may be scraped from the plates and subjected to ultraviolet analysis. Once the absorbance maxima and minima of the rugs are known then the presence of the particular drug can be confirmed by spectrophotometry.

Presence of several drugs such as cocaine, amitriptylene, chlordiazepoxide, chlorpromazine, diazepam, imipramine, and also salicylates, barbiturates can be confirmed by UV spectrophotometry for which the procedure has been already standardized.

Methodology for extracting the organophosphates, barbiturates and salicylates from the affected patients has also been standardized and the same methodology has been adopted for extraction of the toxins from the biological sample. (William , 1991)

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The materials and methodology for the analysis of organophosphates, barbiturates and salicylates have been given in detail in the following section. The methodology involves the following steps.

a) Extraction of the poison from the sample
b) Performing TLC with the extract
c) Visualizing the spot.
d) Performing uv spectrophotometry for to find the maximum absorbance of barbiturates.

(William, 1991)

3.1 ANALYSIS OF ORGANOPHOSPHATES BY TLC:

Materials required:

TABLE 3.1:

Materials	Number
TLC plate	One per patients' sample
Vortex mixer	one
Test tubes	One per sample
Beaker	One per sample

Chemicals required:

1) Chloroform
2) Hexane
3) Dichloromethane

Methodology:

1) Patients' sample was collected.
2) It was mixed with equal amount of chloroform.
3) The mixture was vortexed for 5 to 10 minutes.
4) It was kept in hot air oven for 10-15 min.
5) 10 microlitres of the mixture was taken and spotted on the TLC plate.
6) Then the plate was kept in a closed beaker containing a mixture of the following solvents.

   Acetone- 5ml

   Dichloromethane- 2.5 ml

   Hexane-30 ml

7) The solvent rose due to capillary action. Once the solvent rose up to ¾th of the plate, the plate was taken out of the beaker and the result was visualized under ultraviolet light.

3.2 ANALYSIS OF BARBITURATES AND SALICYLATES BY TLC

Materials required:

TABLE 3.2:

Materials	**Number**
Separating funnels	Minimum 3 per sample
Test tubes	8 per sample
Beaker/conical flask	3 per sample
Test tube stand	One

Chemicals required:

1) Boric acid
2) Potassium chloride
3) Chloroform
4) Animal charcoal(norit-A)
5) Dibasic potassium phosphate
6) Monobasic sodium phosphate(hydrated salt 12 water)
7) Sodium hydroxide
8) Phenobarbital
9) Diphenylcarbazone

Reagents to be prepared:

1) Borate buffer, 0.6M:

Preparation:

37.1 g boric acid and 44.7 g potassium chloride was dissolved in distilled water and diluted to 1L.

2) Phosphate buffer, pH 7.4:

Preparation:

a) For solution A 17.0 g dibasic potassium phosphate was dissolved in distilled water and diluted to 250 ml making a 0.5M solution.

b) For solution B 179.0 g monobasic sodium phosphate (hydrate salt 12 water) was dissolved in distilled water and diluted to 1L making a 0.5M solution. Various hydrated forms of monobasic sodium phosphate are available.

c) pH 7.4 buffer was prepared by mixing 19.2 ml of solution A with 80.2 ml of solution B. A pH meter was used for checking the pH.

3) Sodium hydroxide, 0.45N:

Preparation:

18.0 g sodium hydroxide was dissolved in distilled water and diluted to 1L. It is stored in a polyethylene bottle.

4) Barbiturate control:

a) For stock standard (1mg/ml) 100 mg of Phenobarbital was dissolved in reagent ethanol and diluted to 1 dL with ethanol.

b) For control (10 µg/ ml) 0.10 µl stock standard was diluted to 10 ml.with drug free blood, plasma, or serum if available; alternately, it was diluted with water.

5) Diphenylcarbazone spray:

10 mg diphenylcarbazone was dissolved in 1 dL acetone-water (1:1) before use.

Methodology:

1) 10 ml of water, 10 ml of control and 10 ml of sample was added to three different 125 ml separating funnels.
2) Each of it was extracted three times with 30 ml of chloroform.
3) A few drops of chloroform were added if an emulsion occurred and the separating funnel was inverted gently several times.
4) The combined chloroform extracts were filtered through a thin layer of cotton into a second separating funnel.
5) The chloroform was washed with 5ml of phosphate buffer solution.
6) The chloroform was then drawn off into a 150 ml beaker.
7) The phosphate solution was decanted and it was discarded.
8) The chloroform was then transferred back to the same separating funnel and washed with another 5ml portion of phosphate buffer solution.
9) Step 3 was repeated.

10) The chloroform was added to the separating funnel and 50 mg of norit-A was added to it and shaken vigorously.
11) The solution was filtered through a thin layer of cotton into a separating funnel.
12) 0.45N sodium hydroxide was added and extracted for 5 minutes.
13) Then 0.45N sodium hydroxide was transferred into a clean 15 ml test tube and centrifuged for 5 minutes.
14) The extract was acidified with 50% sulfuric acid and the pH was checked.
15) The unknowns were extracted with 25 ml chloroform and the upper aqueous phase was discarded after centrifugation.
16) The chloroform was washed twice with 10 ml water
17) The water is discarded after each wash.
18) The chloroform was filtered through a thin layer of cotton into evaporation cups.
19) The chloroform was then evaporated to dryness and spotted on TLC plate with barbiturate standard.
20) 1 ml ammonia, 2 ml methanol and 17 ml ethyl acetate together were used as rising solvents.
21) The TLC plate was kept inside a beaker containing the solvent mixture
22) The beaker was closed with a lid and the solvent was allowed to rise on the TLC plate.
23) Once the solvent rose to ¾th of the plate, the TLC plate was taken out and allowed to dry.
24) Next, diphenylcarbazone spray was sprayed on the TLC plate.
25) The result was visualized under u-v light.

Procedure to determine the absorbance maxima of barbiturates:

1) Steps 1 to 13 were repeated as mentioned in the previous procedure.
2) Two test tubes each for blank, control and sample were prepared and the test tube were labeled as follows:

a. NaOH sample (1, 2, etc.)
b. Borate sample (1, 2, etc.)
c. NaOH blank
d. Borate blank
e. NaOH control
f. Borate control

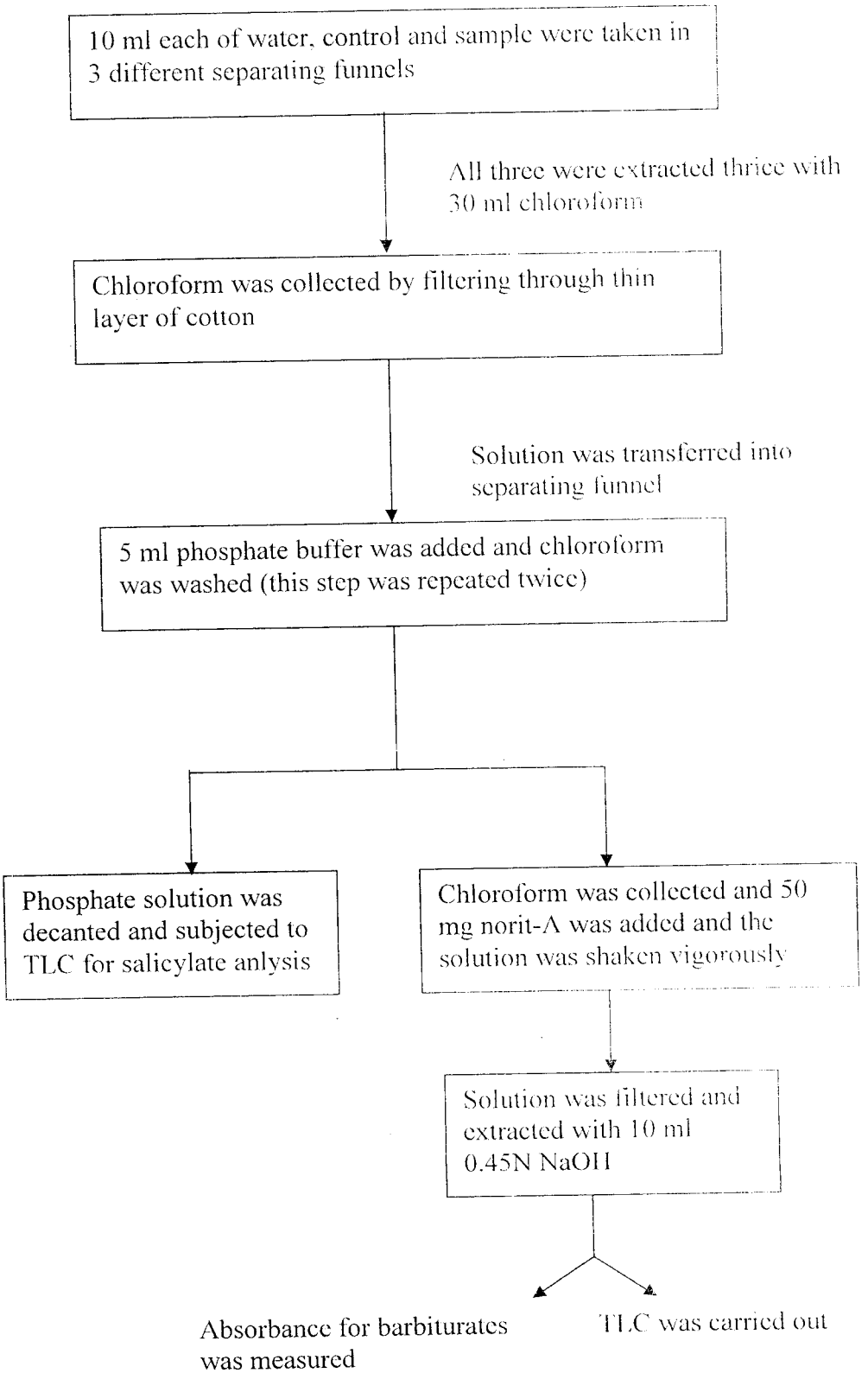
3) 2.0 ml 0.45N NaOH was pipetted into each tube labeled NaOH and 2.0 ml of borate buffer solution into each tube labeled borate.
4) 2.0 ml 0.45N NaOH was pipetted into each tube labeled sample or control and 2.0 ml of sodium hydroxide blank extract was pipetted into each tube labeled blank and the solutions were mixed thoroughly.
5) The ultraviolet spectra of the extracts from 300-200 nm were recorded as follows:
    A. Borate samples and control against borate blank as reference.
    B. Sodium hydroxide samples and control against sodium hydroxide blank.

3.3 UV – SPECTROPHOTOMETRIC ANALYSIS OF BARBITURATES

Methodology:

1) Steps 1-6 were repeated as mentioned in the first procedure.
2) The phosphate solution was decanted into an evaporation cup
3) The solution was evaporated to make it concentrated
4) Then, it was spotted on TLC plate.
5) The TLC plate was then kept in a beaker containing 20 ml ethyl acetacte as rising solvent.
6) When the solvent rose to $3/4^{th}$ of the plate, the plate was removed from the beaker and was allowed to dry.
7) The result was viewed under u-v light.

Flow Sheet depicting extraction of drugs from patient's sample :



RESULTS AND DISCUSSION

4.1 RESULTS FOR OP POISONING

Thirty three samples were analyzed totally for the organophosphates by Thin Layer Chromatography. After the extraction from the gastric aspirates, the patients' sample extract was run along with OP standard on the TLC plate. From table3, it is inferred that three patients tested positive for the presence of Organophosphate. The presence of OP was confirmed from the results of TLC

TABLE 4.1: RESULTS FOR OP POISONING

S. no	Patients' name	Age	Sex	Date of analysis	Result
1	Bharath	21	Male	28.01.2010	-ve
2	Poorani	42	Female	28.01.2010	-ve
3	Dharani	40	Male	29.01.2010	**+ve**
4	Vijayaraj	23	Male	30.01.2010	-ve
5	Meghala	15	Female	30.01.2010	-ve
6	Ganapathy	24	Male	31.01.2010	-ve
7	Rajendran	26	Male	31.01.2010	-ve
8	Haridoss	35	Male	31.01.2010	-ve
9	Ponnusamy	55	Male	01.02.2010	-ve
10	Renuka	25	Female	03.02.2010	-ve
11	Jyothi	17	Female	04.02.2010	-ve
12	Ravi	45	Male	04.02.2010	-ve
13	Pavithra	11	Female	04.02.2010	-ve
14	Benazir	33	Female	04.02.2010	-ve
15	Muthuraj	33	Male	05.02.2010	-ve
16	Samundeshwari	22	Female	08.02.2010	-ve
No sample on 09.02.2010					
17	Girija	20	Female	10.02.2010	-ve
18	Tulasi	20	Female	10.02.2010	-ve

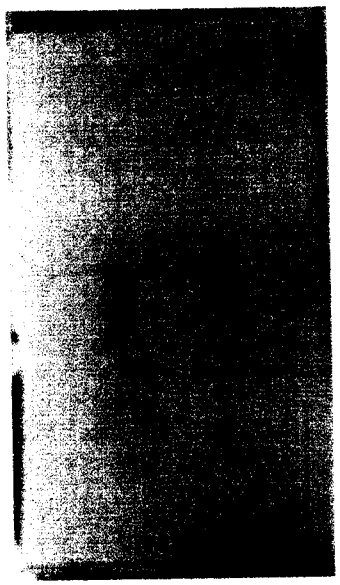
19	Dharman	45	Male	10.02.2010	-ve
No sample on 11.02.2010					
20	Vinodhini	22	Female	12.02.2010	-ve
21	Anbu	18	Male	12.02.2010	-ve
22	Shivakumar	34	Male	15.02.2010	-ve
23	Sheeba	14	Female	15.02.2010	-ve
24	Dinesh	25	Male	15.02.2010	-ve
No sample on 16.02.2010 and 17.02.2010					
25	Bhagyam	40	Female	18.02.2010	-ve
26	Unknown	40	Male	18.02.2010	-ve
27	Karthika	25	Female	18.02.2010	-ve
28	Durai	42	Male	18.02.2010	**+ve**
No sample on 19.02.2010 and 20.02.2010					
29	Ambika	48	Female	21.02.2010	-ve
30	Usha	52	Female	21.02.2010	-ve
31	Vijay	54	Male	22.02.2010	-ve
32	Raghu	42	Male	27.02.2010	**+ve**
33	Sundararajan	52	Male	27.02.2010	-ve

Patient 3- Dharani, patient 28- Durai and patient 32- Raghu showed positive result for OP poisoning.

FIG 4.1 TLC RESULTS FOR OP

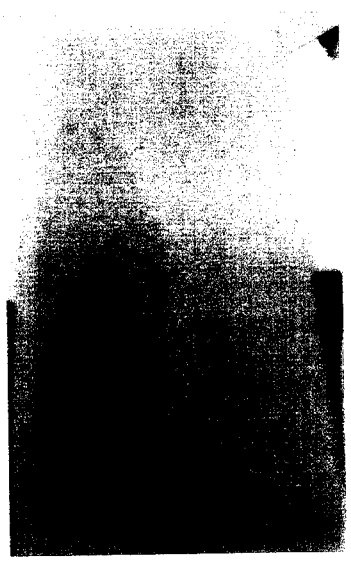
FIG 4.1a

OP positive



Patient 3's sample was spotted on the TLC plate and run along with OP standard. A dull purple color line, that was similar to that of the standard, appeared to the right of the standard where the sample extract was spotted. This indicated the presence of organophosphate in the sample.

FIG 4.1 b
OP negative



Patient 4's sample was spotted on the TLC plate and run along with OP standard. The dull purple line that appeared on the chromatogram of sample 3 was found to be absent in the chromatogram that was developed for sample 4. Absence of the dull purple color line indicated the absence of organophosphate. (Peter et al, 2006)

4.2 RESULTS FOR OVER DOSAGE OF DRUGS:

Patients who were suspected to be affected by over dosage of the drugs barbiturates and salicylates were subjected to gastric aspiration and their aspirate samples were analyzed. Firstly the drugs were extracted from the suspected patients' sample and then TLC was carried out. The extract was spotted on the TLC plate along with respective standards and the results were visualized by spraying Diphenylcarbazone(DPC) on the plate in the case of barbiturates. On the other hand the result is simply visualized under UV for salicylates.(William, 1991)

TABLE 4.2: RESULTS FOR BARBITURATES AND SALICYLATES POISONING

S. no	Name of the patient	Age	Sex	Date	Result for barbiturates	Result for salicylates
1	Soundararajan	52	Male	05.03.2010	**+ve**	-ve
2	Mahalakshmi	46	Female	05.03.2010	**+ve**	-ve
No samples on 06.03.2010 & 07.03.2010						
3	Narayanan	45	Male	08.03.2010	**+ve**	-ve
No samples on 09.03.2010 , 10.03.2010, 11.03.2010, 12.03.2010						
4	Karthiga	16	Female	13.03.2010	-ve	-ve
No samples on 14.03.2010						
5	Prabhu	19	Male	15.03.2010	-ve	-ve
6	Malathi	21	Female	16.03.2010	-ve	**+ve**
No samples on 17.03.2010						

7	Thangam	40	Female	18.03.2010	-ve	-ve
8	Vijaya	28	Female	19.03.2010	**+ve**	-ve
9	Hemavathy	43	Female	19.03.2010	-ve	**+ve**
10	Anandhan	37	Male	20.03.2010	-ve	-ve
No samples on 17.03.2010						
11	Benjamin joseph	25	Male	21.03.2010	-ve	-ve
12	Pradeepa	25	Female	22.03.2010	-ve	-ve
13	Satish	30	Male	22.03.2010	-ve	-ve
14	Girija	35	Female	22.03.2010	-ve	-ve
15	Bhakyaraj	37	Male	23.03.2010	-ve	-ve

From table 4 it can be inferred that totally 15 samples were analyzed for barbiturates and salicylates out of which 4 gave positive results for barbiturates over dosage while 2 gave positive results for salicylates over dosage. The chromatogram for positive and negative indicating the presence and absence of results of barbiturates and salicylates respectively. are depicted in the following figures. (fig 4, 5)

Fig 4.2 TLC results for Barbiturates

FIG 4.2a

Barbiturates positive

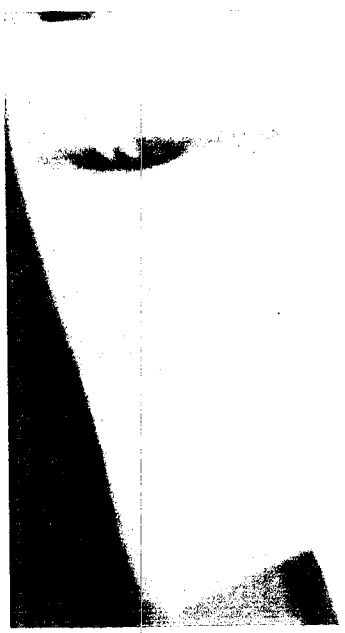


FIG 4.2b

Barbiturates negative

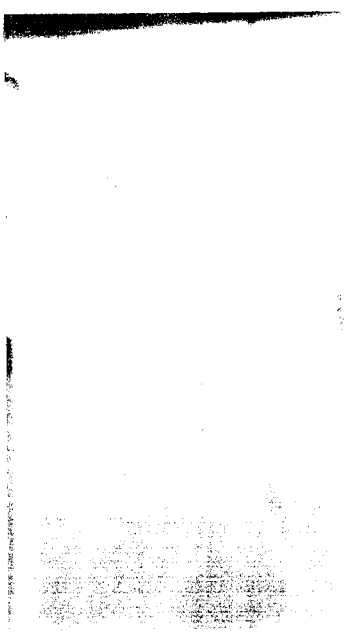


Figure 4a shows positive result for barbiturates when sample 1's extract was spotted along with barbiturate standard on the TLC plate. The dark violet spot indicates the standard while the light violet spot indicates the sample extract whose barbiturate concentration is low when compared to the standard.

Figure 4b shows the absence of barbiturates wherein the light violet color spot is absent. (Moffat et al, 1986)

Fig 4.3 TLC Results for Salicylates

FIG 4.3a
Salicylates positive

FIG 4.3b
Salicylates negative

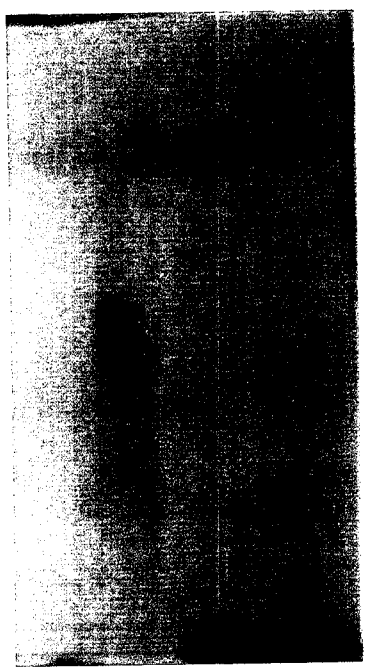


Figure 5a shows two violet color lines, one on side of the other. The first line represents the standard that was spotted while the second line represents the extract showing the presence of salicylates.

In the figure 5b, the line which must show the presence of salicylates is absent. Hence it indicates the absence of salicylates in the sample.

4.3 RESULTS FOR UV – SPETROPHOTOMETRY

The maximum absorbance of barbiturates was found by UV- Spectrophotometry by using the samples that tested positive for barbiturates over dosage and the results were compared with the absorbance of those samples that showed negative results for the same. Results were tabulated based on the figures 6,7,8,9 and 10.

TABLE 4.3 RESULTS FOR UV - SPECTROPHOTOMETRY

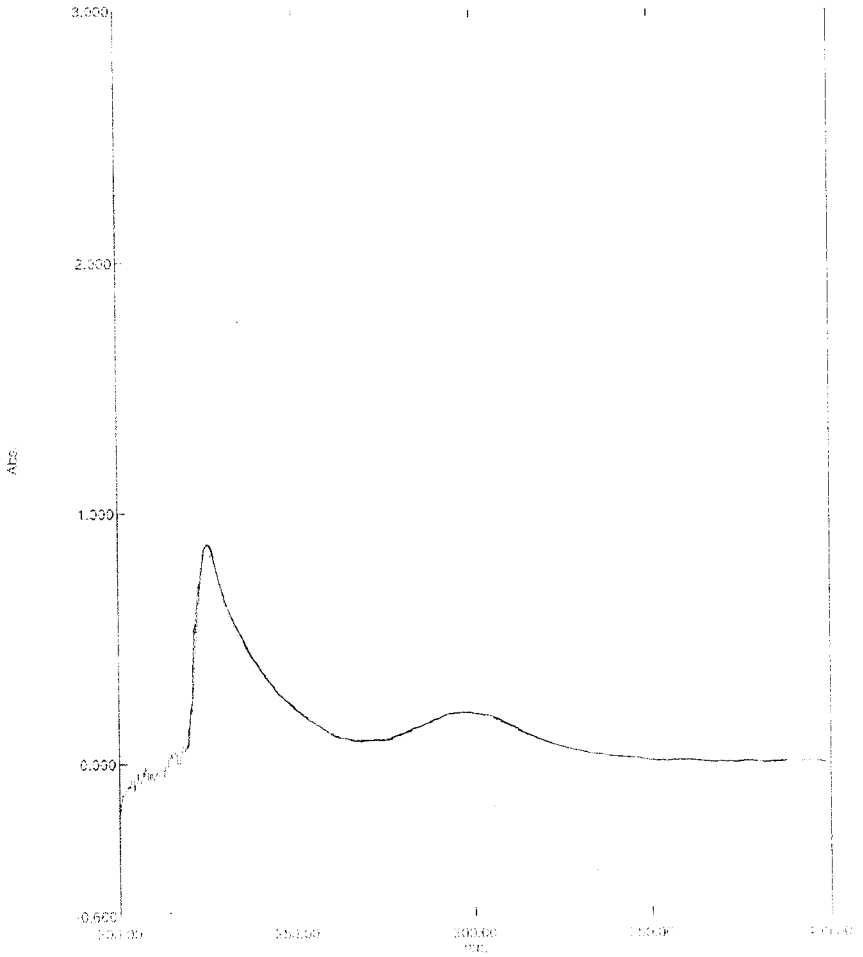
S. No	Patients' name	Result for TLC	Absorbance maxima when NaOH was used as blank		Absorbance maxima when borate was used as blank	
		+ve/ -ve	Wavelength λ (nm)	Absorbance	Wavelength λ (nm)	Absorbance
1	Soundararajan	+ve	297	0.206	296	0.256
2	Mahalakshmi	+ve	300.8	0.551	302.8	0.801
3	Narayanan	+ve	299	2.340	302	2.524
4	Girija	-ve	223.8	1.057	211.8	1.986
5	Thangam	-ve	249	0.119	208	2.385

The absorbance pattern of each sample is depicted in the following figures.(fig 6,7,8,9,10)

FIG 4.4a

Graph Representing Absorbance of sample extract From Patient 1:

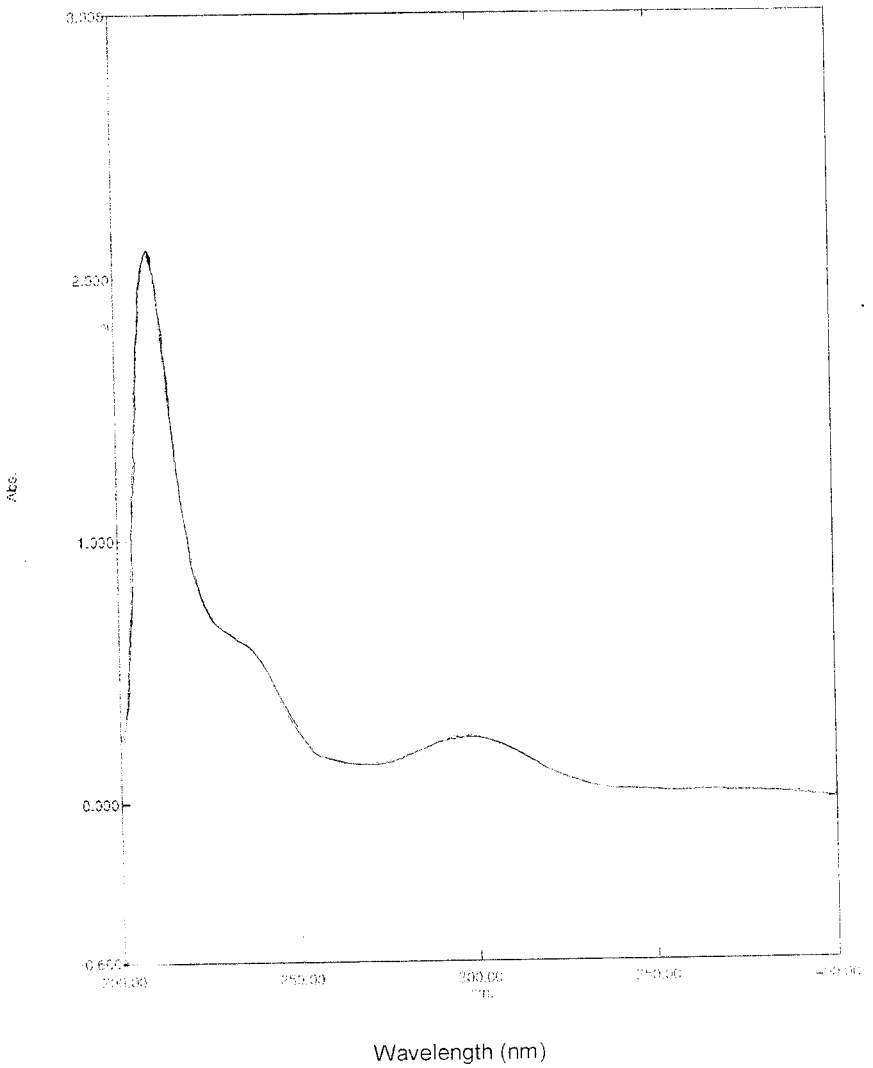
Absorbance maxima when NaOH was used as blank



Maximum absorbance was found to be 0.206 at 297nm when NaOH was used as blank.

FIG 4.4b

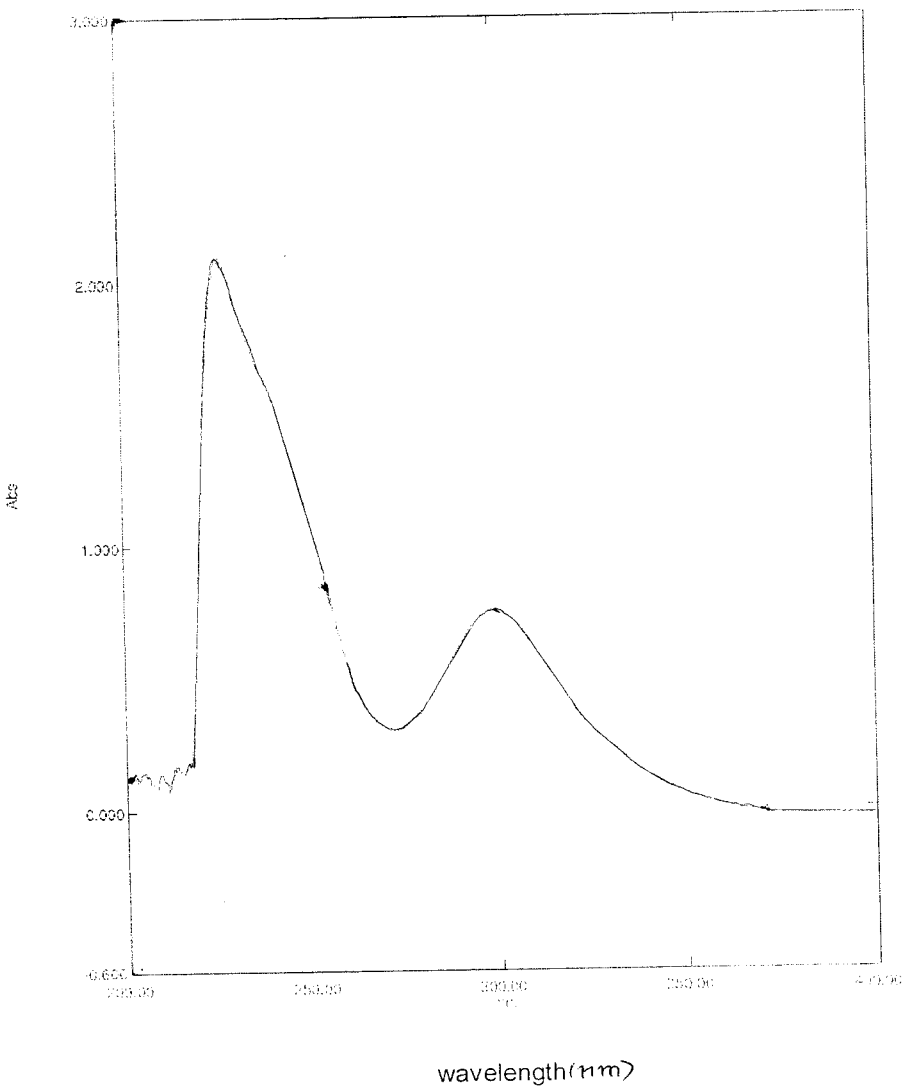
Graph Representing Absorbance of sample extract From Patient 1: Absorbance maxima when Borate was used as blank



Maximum absorbance was found to be 0.256 at 296nm when borate was used as blank.

FIG 4.5a

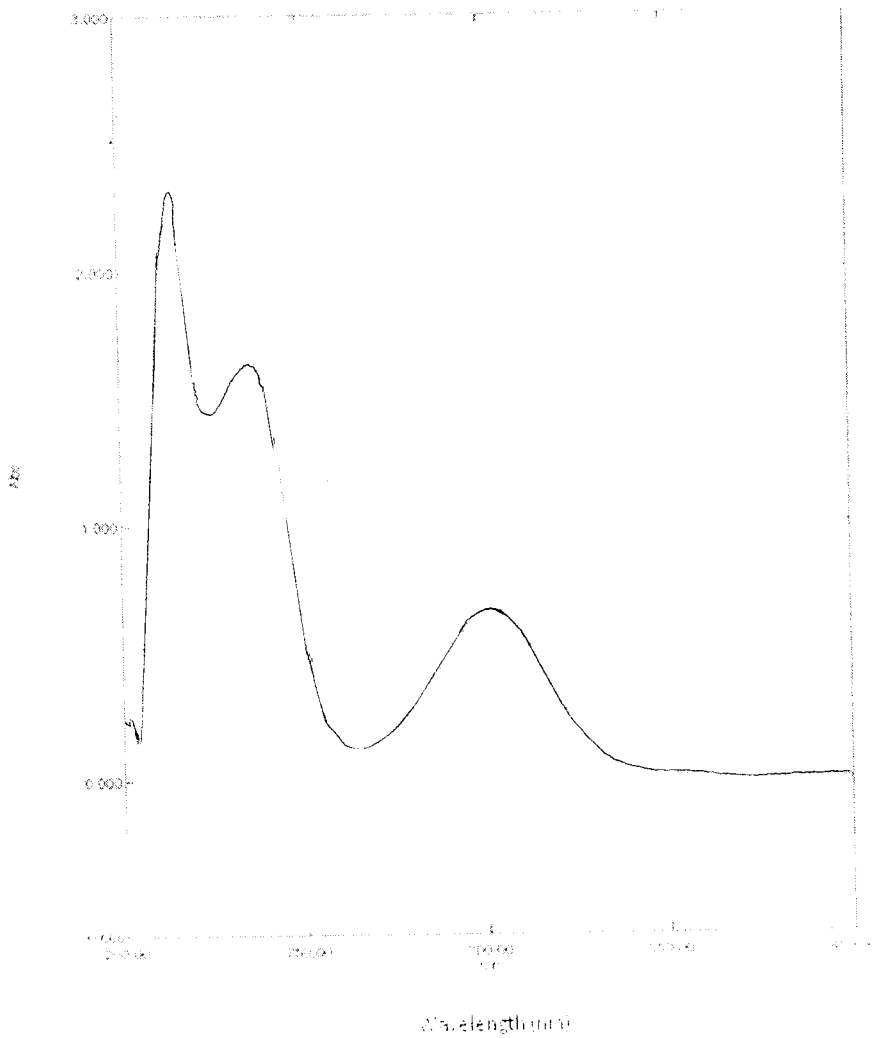
Graph Representing Absorbance Of sample extract from Patient 2: Absorbance maxima when NaOH was used as blank



From figure 7a, it is seen that the maximum absorbance was 0.881 at wavelength 300.8nm , for sample 2.

FIG 4.5b

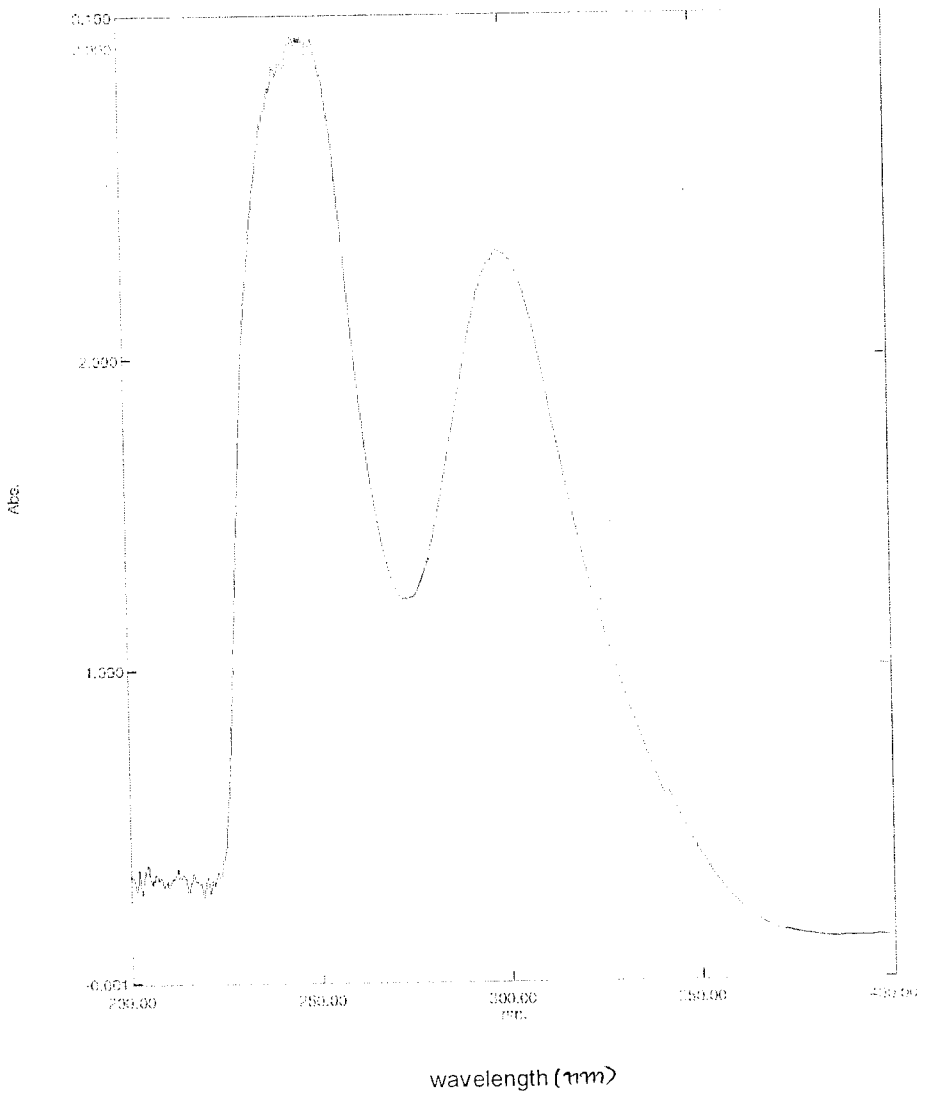
Graph Representing Absorbance Of sample extract from Patient 2: Absorbance maxima when Borate was used as blank



0.801 was found to be the maximum absorbance value, for sample 2, at a wavelength of 302. 8nm, when borate was used as blank.

FIG 4.6a

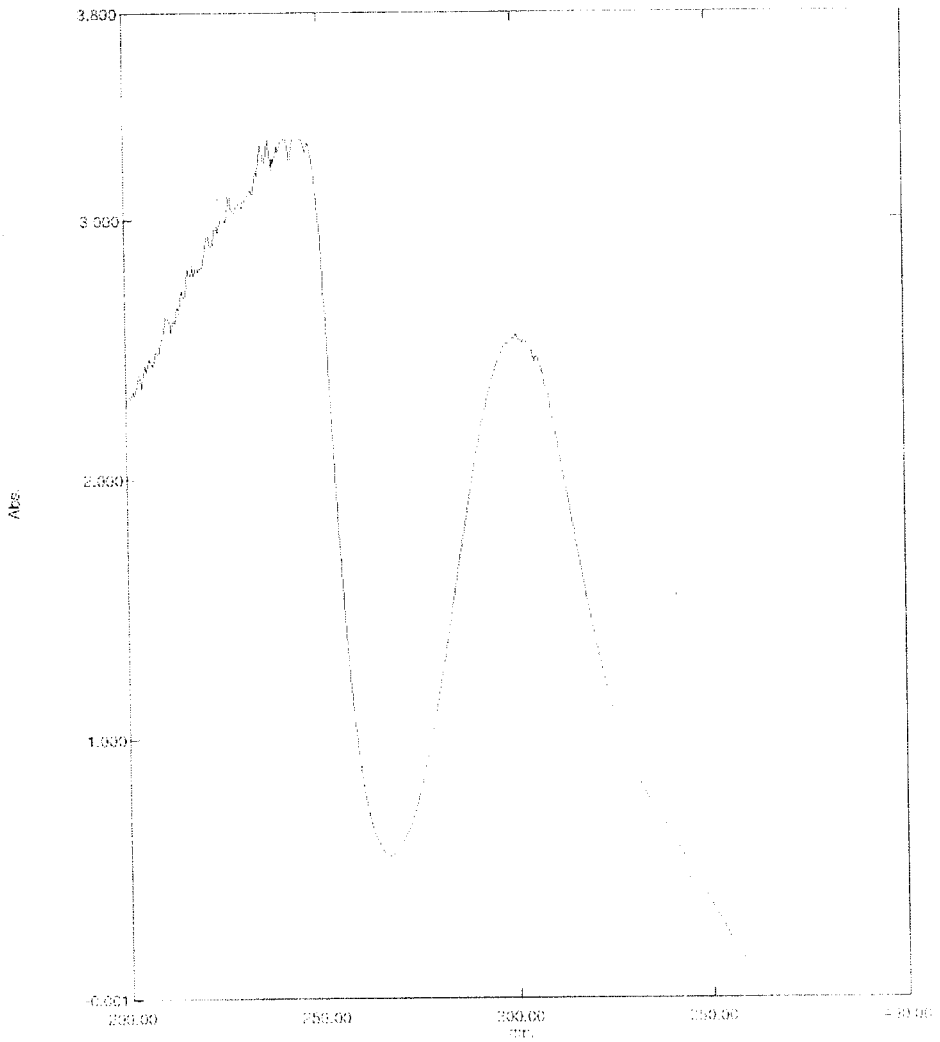
Graph Representing Absorbance of sample extract From Patient 3: Absorbance maxima when NaOH was used as blank



2.340 was found to be the maximum value for absorbance, for sample 3,at a wavelength of 299nm, when sodium hydroxide as used as blank.

FIG 4.6b

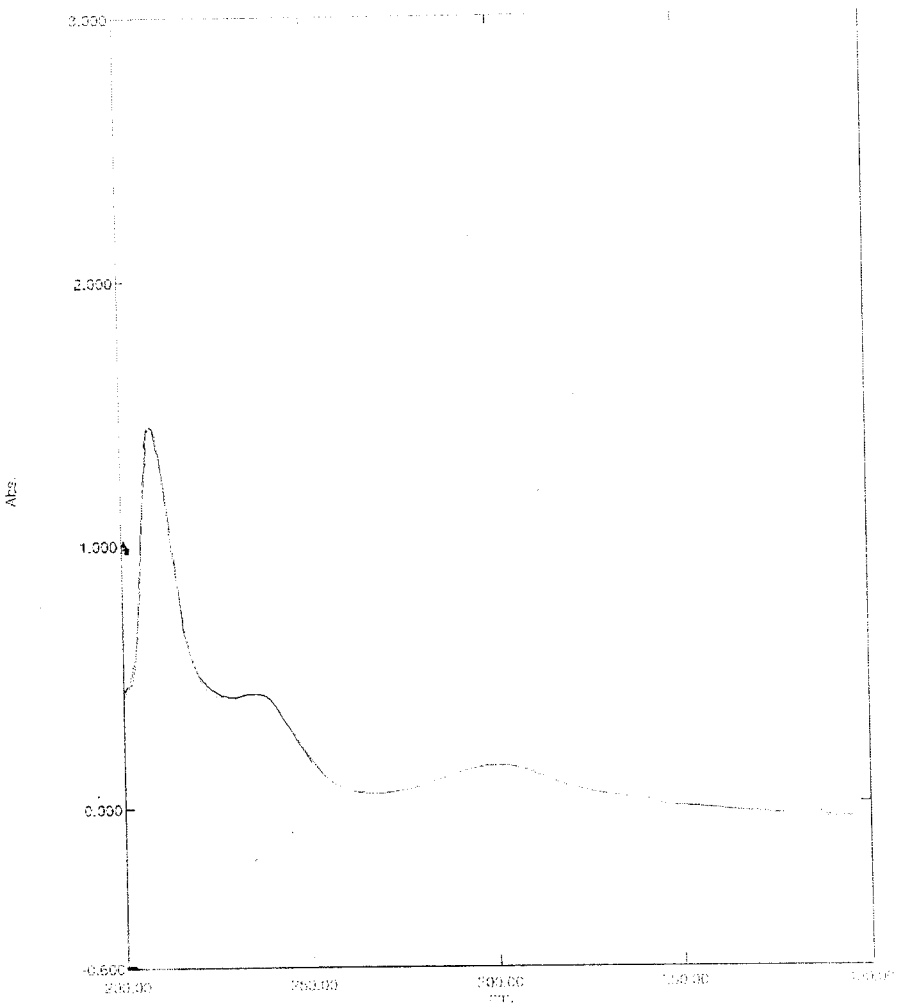
Graph Representing Absorbance of sample extract From Patient 3: Absorbance maxima when Borate was used as blank



2.524 was found to be the maximum value for absorbance, for sample 3, at a wavelength of 302nm, when borate was used as blank.

FIG 4.7a

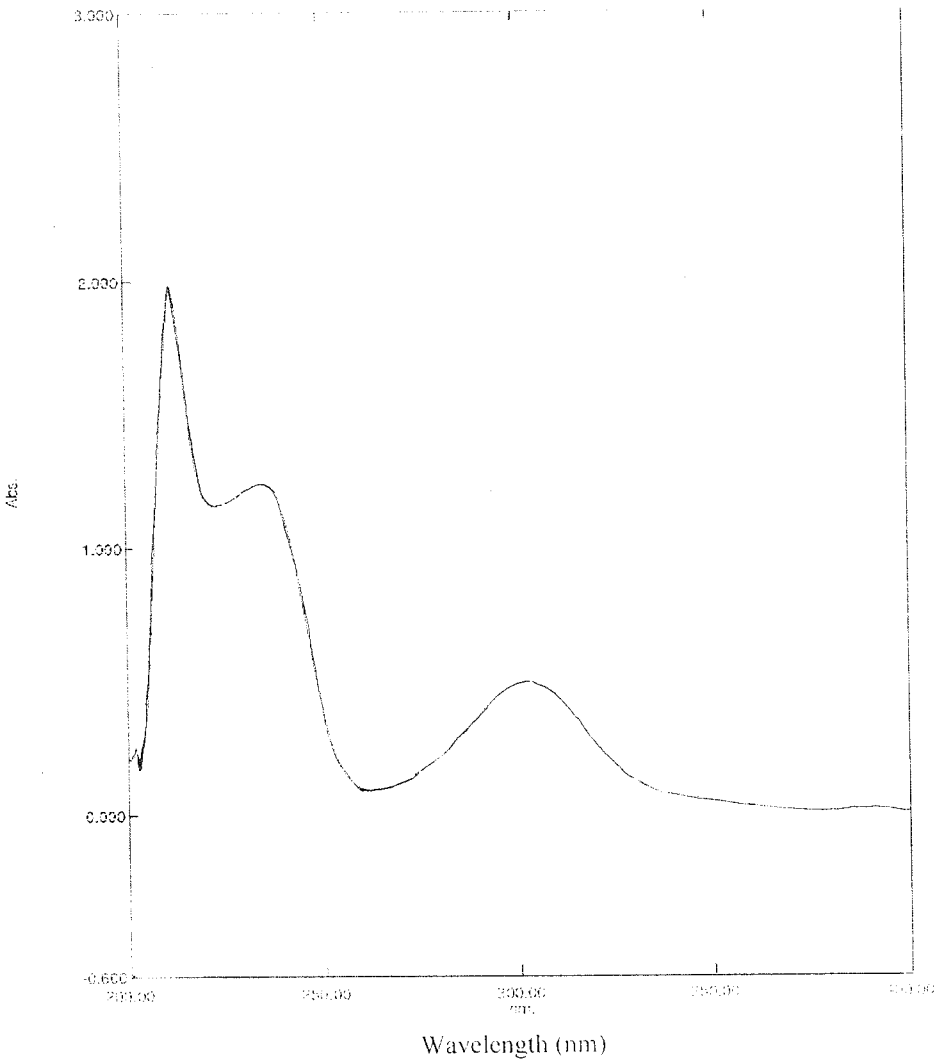
Graph Representing Absorbance of sample extract From Patient 4: Absorbance maxima when NaOH was used as blank



From the above figure, 1.507 was found to be the maximum absorbance value at a wavelength of 223.8nm, when NaOH was used as blank.

FIG 4.7b

Graph Representing Absorbance of sample extract From Patient 4: Absorbance value when Borate was used as blank



Wavelength (nm)

From the above figure, 1.986 was found to be the maximum absorbance value at a wavelength of 211.8nm when borate was used as blank.

FIG 4.8a

Graph Representing Absorbance of sample extract From Patient 5: Absorbance maxima when NaOH was used as blank

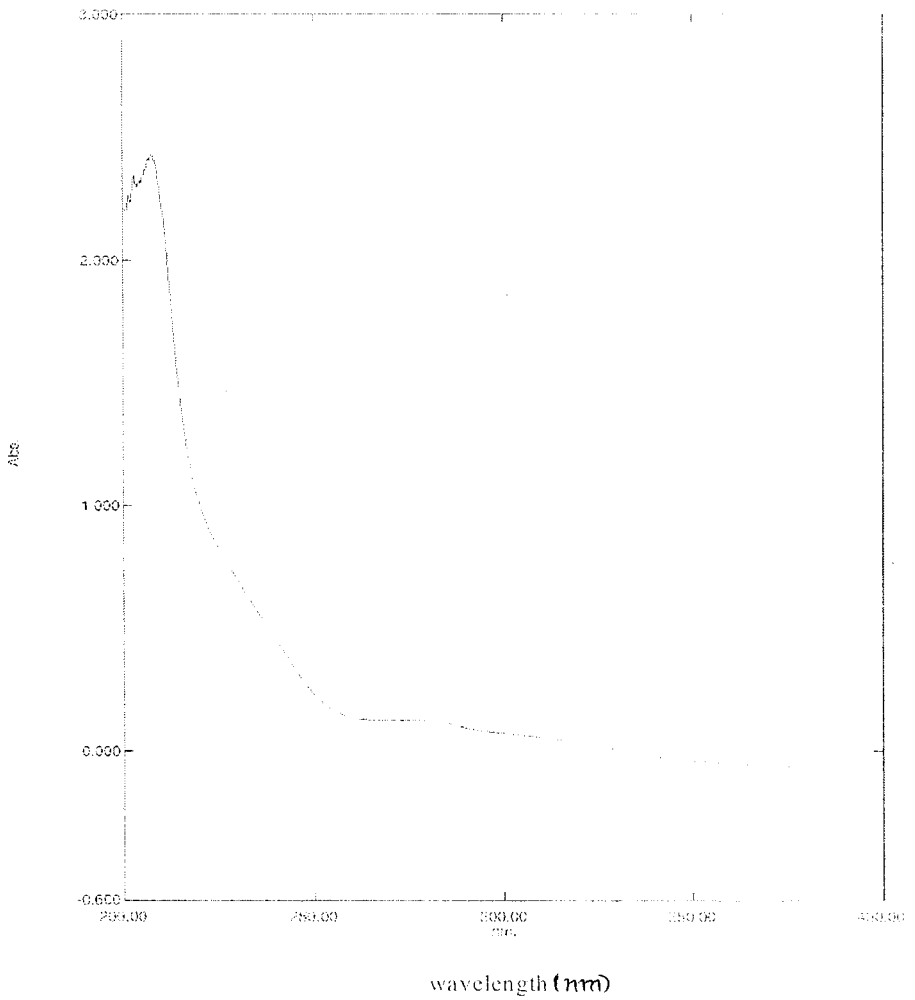


Figure 10b shows 0.119 as the maximum absorbance for sample 5, at a wavelength of 249 nm, of 249nm when NaOH was used as blank

FIG 4.8b

Graph Representing Absorbance of sample extract From Patient 5: Absorbance Maxima when borate was used as blank

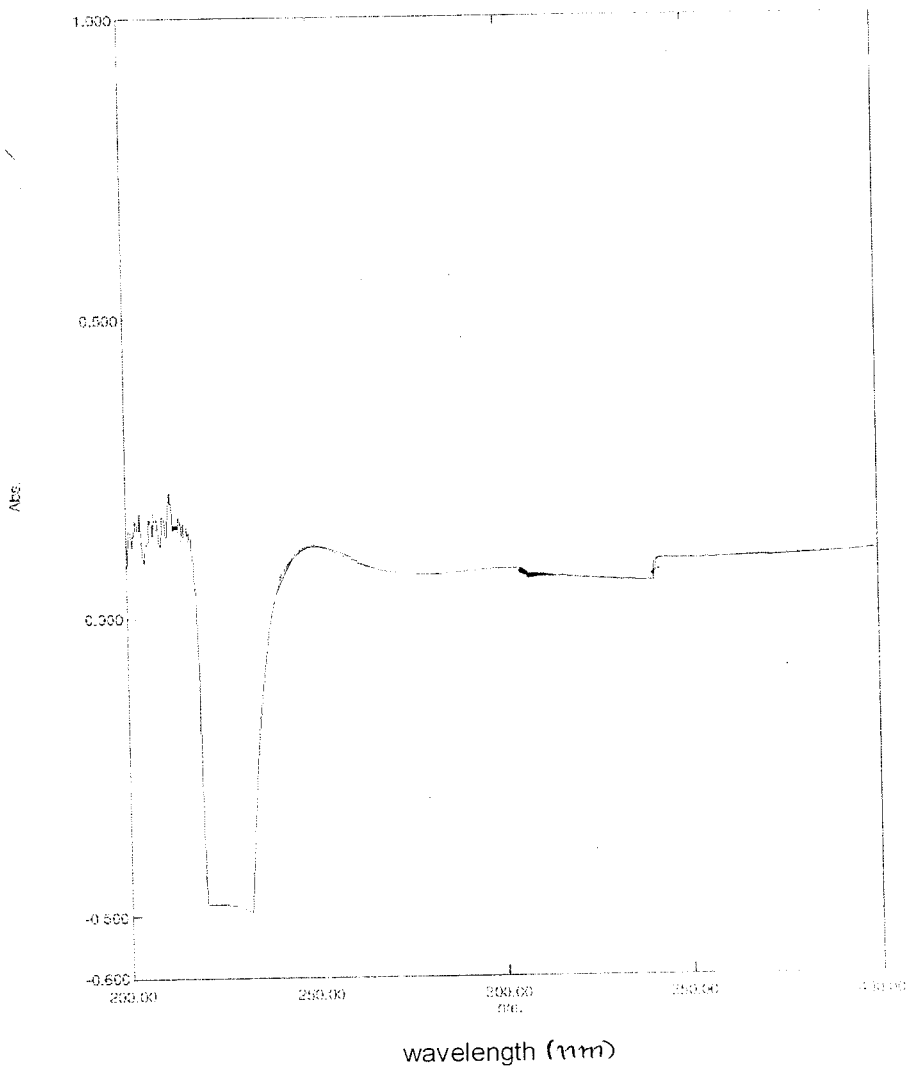


Figure 10b shows 2.385 as the maximum absorbance for sample 5, at a wavelength of 208nm, when borate was used as blank.

From the above results the following was inferred:

- The wavelength at which the sample extracts, that showed positive results for barbiturates over dosage, showed maximum absorbance was close to 300nm.
- It was also noted that the normal samples (which has no babiturates) showed maximum absorbance at a different wavelength when compared to barbiturates positive samples.
- A considerable variation was also found in the absorbance values, both when NaOH and borate were used as blank.

CONCLUSION

5. CONCLUSION

Out of the 33 samples that were analyzed, 3 tested positive for organophosphates. For the drugs of class barbiturates and salicylates, 14 samples were analyzed out of which 4 samples tested positive for barbiturates and 2 samples tested positive for salicylates.

CONCLUSION 1:

Majority of the samples showed negative results due to two reasons.

- One reason is due to the presence of some other toxic compound other than OP or due to the over dosage of some other class of drugs. In this case the doctors treat the patients by ruling out the treatment for OP/ barbiturates/ salicylates and provide some other treatment which is suitable for the patient.
- Delayed arrival of patients at the hospital also account for the negative result. This is because the pesticide or the drug would have got absorbed into the blood. Hence the gastric contents of the patients will not contain any poison (here the poison being OP/ barbiturates/ salicylates). Thus negative result is obtained in this case when TLC is carried out.

CONCLUSION 2:

The maximum absorbance was found for three samples that tested positive for barbiturates and also for 2 samples that tested negative for barbiturates using u-v spectrophotometry technique. Since very few samples were analyzed using u-v spectrophotometer and the absorbance maxima for each sample was found, definite conclusions cannot be drawn based on the inferred results.

CONCLUSION 2:

Organophosphates, one of the main classes of pesticides affect the nervous system variety of symptoms like paralysis or weakness. On the other hand, over dosage of barbiturates and salicylates are also proven to produce toxicity in the biological system. Patients

affected by these poisons arrive at the hospitals in the unconscious or semi- conscious state. Hence it becomes vital to treat the patients at a faster rate and in an efficient way. There are various chromatography techniques like HPLC, Gas Chromatography, etc that are employed to identify the poisons present in the patients' sample. But these techniques have proven to be expensive and hence they cannot be set up in small scale laboratories and hospitals. As an alternative, Thin Layer Chromatography is employed for analyzing the samples. This technique is simple and cost effective when compared to the other techniques. It also gives accurate and reliable results and thus it is being employed in the hospitals and labs.

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6. REFERENCES

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