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Thin Layer Chromatographic Detection of Dicyclomine Hydrochloride Extracted from Whole Blood

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Abstract

Dicyclomine Hydrochloride belongs to a class of synthetic anticholinergics. It is regularly used for the treatment of spasmodic pain, spasmodic dysmenorrhea, and renal, ureteric and biliary colic. This drug is easily available in the market and can be abused for suicidal purposes. Its unsupervised use can lead to abuse, accidental poisoning along with the occasional report of intentional suicidal poisoning. Hence its analysis is very important for medicolegal purposes.Biological samples of choice for routine qualitative analysis of drug is liver, stomach content, whole blood, and urine. Routine toxicological procedures employ highly sophisticated state of the art instruments like High Performance Liquid Chromatography (HPLC), Gas Liquid Chromatography (GLC) and Liquid Chromatography-Mass Spectrophotometer (LC-MS) for the estimation of drug from biological and non-biological matrices but the present paper deals with an attempt to analyse the drug extracted from the biological sample of interest using a simple, feasible and optimal method with the help of Thin Layer Chromatography (TLC).

The study involves the extraction of the drug from matrix (blood), using Liquid-Liquid Extraction method of alkaline plasma followed by its separation and identification by Thin Layer Chromatography. The present paper describes a simple, economical, reproducible sample extraction, clean-up and detection method that can be easily attempted in a laboratory.

Keywords: Whole blood; Dicyclomine; Extraction; Analysis; Drug.

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Introduction

Dicyclomine Hydrochloride, also available as Dicycloverine®; is chemically 2-Diethylaminoethyl 1-cyclohexylcyclohexane-1-carboxylate hydrochloride, with structure shown in Figure-1. Dicyclomine is a carboxylic acid derivative and a selective anticholinergic with antispasmodic activity. It is regularly used for the treatment of spasmodic pain, spasmodic dysmenorrhea, and renal, ureteric and biliary colic.[1]

The drug was first synthesised chemically for use in the United States by the scientists of William S. Merell Company in 1949(2), and FDA approval in US on 11th May 1950.

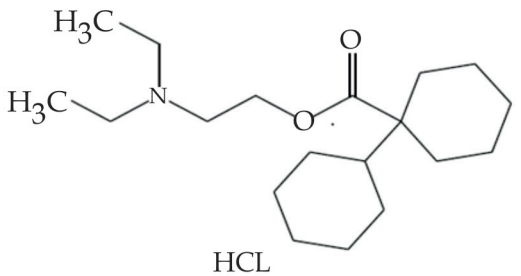

Fig. 1: Chemical structure of Dicyclomine Hydrochloride.

Commercially, Dicyclomine is available as Bentyl; Colimon; Drovid; Declor-20; dicyclomine hydrochloride injections and many other formulations. This drug is easily available over-

the-counter as a painkiller in markets in India. Pharmaceutical preparation for oral administration, Declor-20® contains 20mg of Dicyclomine hydrochloride in each tablet. It occurs as a fine, white, crystalline, practically odourless powder with a bitter taste. It is soluble in water (0.00327mg/ml), freely soluble in alcohol, chloroform, and very slightly soluble in ether.[3] Chemically, the drug has a pKa value of 8.96 making the drug highly basic.

Pharmacologically, Dicyclomine is rapidly absorbed after oral administration through gastrointestinal tract, reaching peak values within 60-90 minutes. Plasma half-life reaches peak value in about 1.8hrs after consumption.[4] The volume of distribution in body for a 20mg oral dose is 3.65L/kg. The principal metabolisis occurs in liver and primary route of elimination is via urine (79.5%) and little in faeces (8.4%).[5] The drug blocks acetylcholine from binding to muscarinic receptors in smooth muscles of the GI tract, relaxing the smooth muscles. The drug and/or its metabolites can be detected in the urine within 1 hour after oral ingestion. The signs and symptoms of over dosage are headache; nausea; vomiting; blurred vision; dilated pupils; hot, dry skin; dizziness; dryness of the mouth; difficulty in swallowing; and CNS stimulation.[6]

Although reports of death by Dicyclomine ingestion are rare, majority being related to infant deaths; reports of abuse are plenty(7–11). The drug is frequently prescribed by general practitioners, physicians and gastroenterologists for treatment of colicky and spasmodic pain.

Blood is the preferred sample for drug estimation in both ante-mortem and post-mortem testing owing to the fact that it is the transporter of vital substances and movement of drug in the body is primarily done by blood but the disadvantage is that it cannot be injected directly in to the analytical instrument, and needs to be prepared and modified according to instrument for analysis.[1214] Therefore the blood sample containing the drug is subjected to pre-treatment which involves isolation of the analyte from matrix, dissolution of extract in a suitable solvent and finally pre-concentration of the analyte of interest in a sequential manner.

Literature review for the last decade has revealed a variety of instrumental and chemical techniques for the identification and quantification of Dicyclomine in bulk, pharmaceutical preparations and human plasma using HPTLC(15–19); HPLC(20–25); and UV(26,27). Dicyclomine has also been analysed quantitatively in human plasma by Capillary Gas Chromatography with Nitrogen-Selective Detection.[28,29] Although these methods are sensitive, they require higher expertise at instrumental assay whereas Thin Layer Chromatography can be utilised as a preliminary sample purification as well as qualitative testing due to its simplicity and time required for analysis.

The present paper has presented an attempt to analyse Dicyclomine Hydrochloride extracted from post-mortem blood and analysed by Thin Layer Chromatography which is comparatively simple, rapid, cost effective and can be performed as a preliminary test as compared to higher instrumentations. The analyte was isolated and cleaned up after liquid-liquid extraction from whole blood to yield a desired amount.[30] Identification was done using different chromogenic reagents. The extraction methodology followed increased the recovery from which spectrophotometric, chromatographic and other instrumental techniques can be used for qualitative and quantitative estimation.

Material Required

Chemicals/Reagents: Acetone; Ammonia; Acetic Acid; Chloroform; Diethyl Ether; Ethyl Acetate; Methanol; Anhydrous Sodium Sulphate; Sodium Tungstate; 0.1N Sulphuric Acid; Toluene.

Ultra-pure Water was produced from Rions (India) Water purification system.

Standard drug: Fixed dose drug (Dicyclomine Hydrochloride) of strength 20mg was procured from local market.

TLC plate: TLC plates of pre-coating Silica gel 60 F254from Merck, Germany.

Glasswares: Chromatographic Chamber; Separating funnel; Volumetric flask; Graduated fine capillary tubes (20µl), Beaker.

Miscellaneous: 15mlsample storage tubes; Whatman filter paper-42; Micropipette (100-1000µl), (20-200µl); Micropipette tips (100-1000µl), (20-200µl).

Methodology

Preparation of stock solution from Certified Reference Material.

- 10mg of standard was taken in a graduated cylinder and final volume made upto 10ml using Chloroform.
- The freshly prepared standard solution is vortexed and stored at 4°C till further requirement.

Preparation of working standard from pharmaceutical preparation

- Working standards were extracted from the pharmaceutical preparation of drug, Dicyclomine hydrochloride.
- 10 tablets with average weight of were crushed to a fine powder in a clean porcelain mortar.A quantity (100mg) of powder was transferred into a 25ml volumetric flask. 20ml of Chloroform was added to the flask and stirred using vortex till a clear solution was obtained.
- This mixture was filtered using Whatman filter paper no.42. The filtrate was air dried completely and then made up by adding Methanol.
- 100ppm solution was made by dissolving 1ml of 1000ppm standard in 10ml of Methanol.

Spiking of Standard in Whole Blood

To 5ml of whole blood, 5ml of 500ppm standard solution was spiked, the mixture vortexed and stored at 40C.

Extraction of Dicyclomine from Whole Blood

The methodology for extraction of spiked sample and clinical sample has been shown in Figure-2 as a flow diagram.

Sample clean-up

- Extracted sample was passed through a chromatographic column filled top to bottom with layers of silica gel (5cm), activated charcoal (2.5cm) and anhydrous sodium sulphate (2.5cm).
- Before clean-up, the column was condition with 10ml Chloroform: Ether (1:3).
- The residue of organic extract was passed through the column and filtrate was collected, this filtrate was further used for analysis.

Activation of TLC Plates

Precoated TLC plate was activated by heating at 1100C for 10min.

Spotting of Standard and Sample on TLC plates

20µl of extract, working standard and standard CRM was spotted consecutively on the plate using graduated capillaries with appropriate markings.

Preparation of Visualising Agents

After resolution of spots from the spotted extract, air dried plateswere sprayed with spray reagents.

A. Iodine fuming

Put few crystals of Iodine crystals in sealed glass chamber, till vapours saturate the chamber.

B. Dragendorff Reagent

Solution-1: Weigh 0.85g of Bismuth Subnitrate, dissolve in 10ml of Acetic Acid and add 40ml of distilled water.

Solution-2: Weigh 8g of Potassium Iodide, and dissolve in 20ml of distilled water.

Mix 5ml of Solution-1, 5ml of Solution-2, 20ml of Acetic Acid, and final volume of 100ml made up with Distilled water before use.

C. P-dimethylaminobenzaldehyde

Dissolve 1gm of P-dimethylaminobenzaldehyde

D. Citric acid in acetic anhydride

Dissolve 2gm of Citric acid in 100ml of acetic anhydride

E. Ninhydrin Reagent

Mix 100mg of Ninhydrin crystals in 25ml of Acetone.

Chromatographic conditions

Spotted plate was developed in different binary and tertiary solvent systems taken in ratios shown in Table 01.

After development, plates were air dried and observed under short (254nm) and long wave (366nm) Ultraviolet Light. Out of various spray reagents specific for detection of amines following reagents as shown in Table-02 were optimised for identification of drug on TLC plate.

Result

The method of chromatographic separation of the drug Dicyclomine hydrochloride using Thin Layer Chromatography is found to be suitable, for analysis in blood due to its versatility, sensitivity and fast speed of qualitative analysis. The analysis involves various parameters to be optimised including appropriate mobile phased and visualizing agent for achievement of higher separation and resolution.

Mobile phase optimization

An exhaustive search of literature revealed different proportions of solvents methanol, chloroform, toluene, ethyl acetate and acetone to be used as mobile phase. Modifiers like formic acid, glacial acetic acid, and ammonia were used to achieve better resolution and compact spots, based on reported literature, several mobile phases

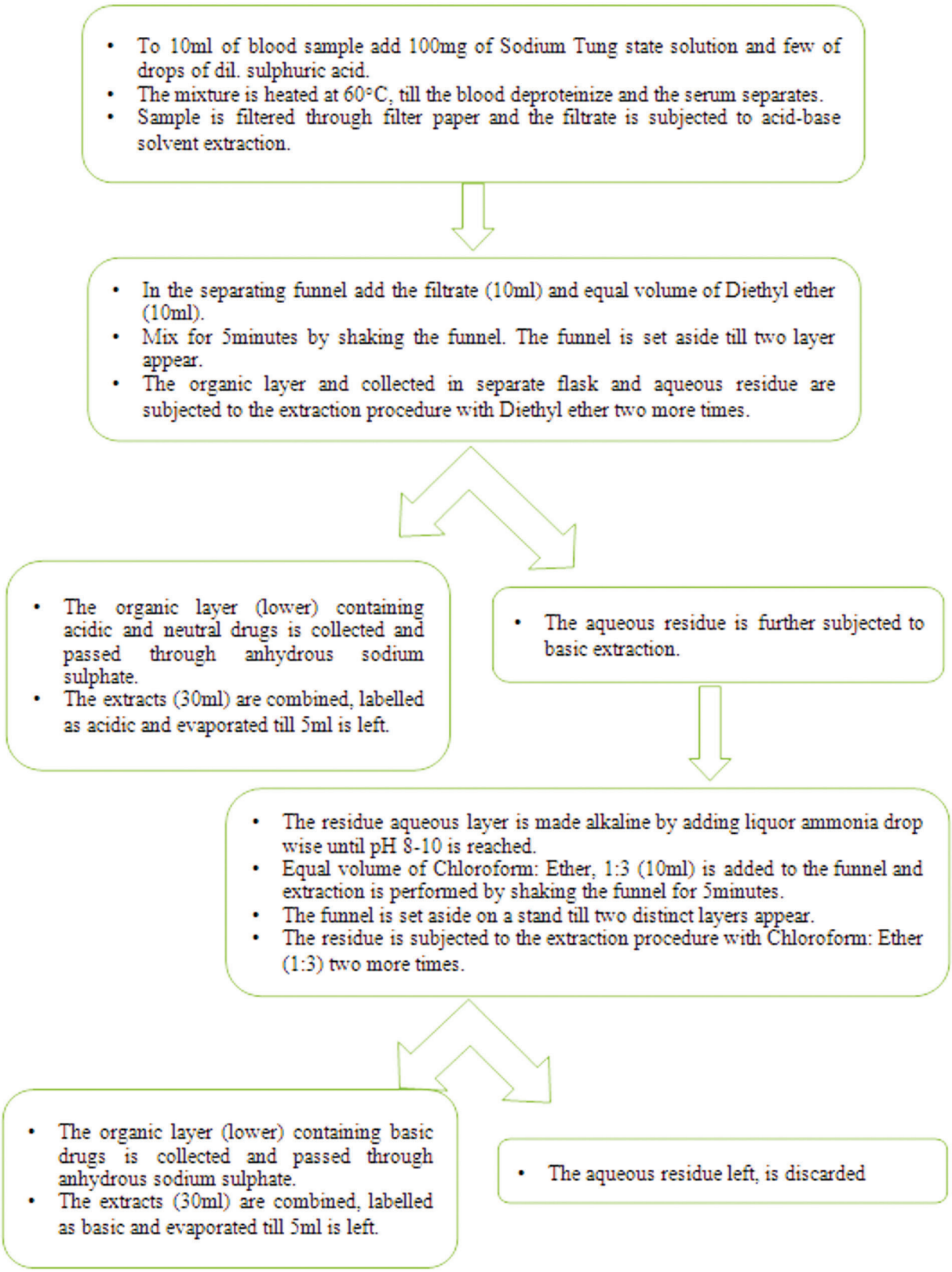

Fig. 2: Schematic Diagram of the Acid-base Extraction Procedure.

were tried as shown in Table 01. The mobile phase, Methanol: Water: Acetic Acid (8:2:0.1) gave the best resolution with the R_f of 0.79.

Table 1: Binary and tertiary solvent systems and their respective Rf.

S. no.	Solvent System	Ratio	Rf
1.	Toluene: Acetone: Formic Acid	10: 9.8: 0.2	0.97
2.	Chloroform: Methanol	9: 1	0.62
3.	Toluene: Acetone: Ammonia	7: 2.5:0.5	0.75
4.	Toluene: Acetone: Methanol: Ammonia	7: 1.5: 1: 0.5	0.90
5.	Chloroform: Acetone	8: 2	0.45
6.	Chloroform: Acetone	6: 4	0.70
7.	Toluene: Acetone: Formic Acid	5: 4.5: 0.5	0.53
8.	Methanol: Water : Acetic Acid	8: 2: 0.1	0.79
9.	Ethyl acetate: Methanol: Ammonia	8.5: 1.0:0.5	0.65
10.	Methanol: Water	8.5:1.5	0.98

Table 2: Various visualization reagents and their respective observations.

S. no.	Spray Reagent	Used for	Expected	Response
1.	Iodine Fuming	Universal Reagent for detection of organic compounds	Yellowish brown spots	Brown spots
2.	Dragen dorff	Detection of nitrogenous alkaloids by forming an ion pair	Orange spots	Orange spots
3.	Ninhydrin	Detection of amino acids, amines and amino sugars	Reddish spots	No response
4.	P-dimethy lamino benzaldehyde	Detection of amines and indoles derivatives	Red spots	No response
5.	Citric acid in acetic anhydride	Detection of tertiary amines	Red or purple spots	No response

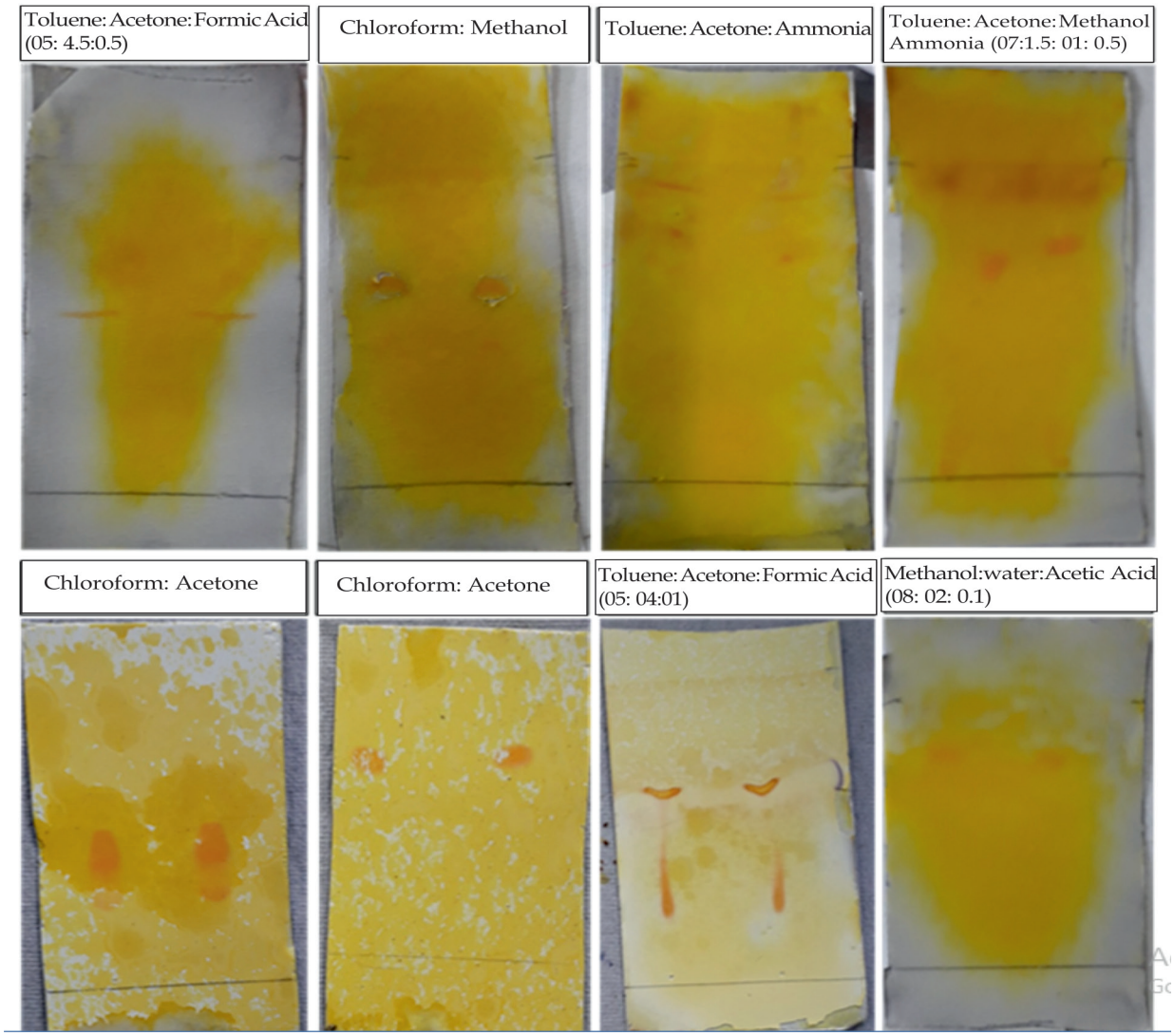

Fig. 3: Results of various mobile phases and visualizing agent (Dragendorff's Reagent).

Selection of visualization agent

A large number of staining reagents were tried i.e. Iodine fuming, Dragendorff's reagent, Ninhydrin, P-dimethylaminobenzaldehyde and solution of citric acid in acetic anhydride. Among these staining reagents, Dragendorff is found to be the best suitable for detection of dicyclomine and gave good results from samples extracted from biological matrix. The results for various mobile phase and visualization reagents is shown in Figure 3.

Discussion

Dicyclomine is a basic drug which is rapidly absorbed in systemic circulation after oral administration through gastrointestinal tract, reaching peak plasma values within 60-90 minutes. The primary route of elimination is via urine (79.5%) and little in faeces (8.4%). For analysis on biological samples, blood and urine are the first choice of matrix irrespective of ante-mortem or post-mortem examination. For the study, methodology for extraction form whole blood was optimised and used to extract analyte from sample received after autopsy for detection of drug.

The present study is based on isolation, extraction and clean-up of Dicyclomine Hydrochloride from blood specimens using conventional Liquid-Liquid Extraction. The LLE works on the principle of solvent extraction and partitioning based on their relative solubilities in two different immiscible liquids, usually water (polar) and an organic solvent (non-polar).[31] Acid-base extraction is therefore a type of liquid-liquid extraction used to separate organic compounds from one layer based on their acid-base properties. The method works on the assumption that most organic compounds are more soluble in organic solvents than in water.

However, if the organic compound is rendered ionic, by changing the pH, it becomes more soluble in water than in the organic solvent. These compounds can easily be made into ions either by adding a proton (an H+ ion), making the compound into a positive ion, or by removing a proton, making the compound into a negative ion. In forensic labs, acid-base extraction is commonly used and done using, diethyl ether, chloroform as organic solvents for extraction.[32]

Sample clean-up was done using column chromatography. Sample clean-up in biological samples, works by eliminating coagulated proteins, large amount of fat molecules and other interferents from the extracted residue that can interference with analysis of target drug. Therefore, we have adopted a simple, precise, economical extraction and clean-up process that gives high quality yield of analytes from spiked and clinical blood samples.

The separation of analyte from matrix was done on a polar adsorbent of Silica gel composed on silicon-oxygen bonds on the surface. The silica was pre-mixed with a fluorescent material F254 that results in absorbance of light at 254nm and emission in the visible spectrum. The detection works by absorption of all light at 254nm and portion of emission obstructed only where the compound is located, making the spots appear dark. Silica gel F254 plates are generally universal, working with wide variety of detection techniques. If the compound in question does not absorb UV radiation, the plates can work with variety of derivatization and spray reagents to form coloured complexes for visualization by naked eye.

As the drug dicyclomine is UV active, the spots were visible in UV light. But for calculation of Rf and spots were sprayed with various spray reagents forming coloured complex with the target analyte.This polar stationary phase is paired with combinations of relatively polar and non-polar mobile phases resulting in separation of analyte from matrix and co-interferents.

Dragendorff is a chemical reagent used to detect tertiary amines and produce an orange or orange pink spots on TLC. During the reaction with drug, the heavy metal atom of (BiI4)i.e. Tetraiodobismuthanuide in reagent combines with nitrogen present in the alkaloid, gets protonated in the presence of acid to form an ion- pair complex which is insoluble orange or red or yellow coloured precipitate. The reagent is a freshly prepared solution of potassium bismuth iodide prepared from basic bismuth subnitrate and potassium iodide in the presence of acetic acid and water.

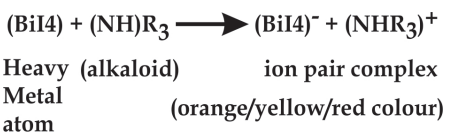

Fig. 4: Reaction of drug with the visualization reagent.

The conditions and reagents used in the proposed methodology are cheap, regularly available in toxicology laboratory and do not require any major sample preparation. Hence, the methodology can be used for routine detection of dicyclomine in standard and extracted samples from whole blood.

Conclusions

Dicyclomine was extracted from spiked and post-mortem whole blood using liquid-liquid extraction method and analysed using pre-coated Thin Layer Chromatography. For chromatographic separation, various binary and tertiary solvent systems were used as mobile phases. Developed plates were viewed under UV light followed by spray of chromogenic reagents which successfully increased the sensitivity without interfering with the simplicity of the method. Solvent systems, showing clear spots of Dicyclomine Hydrochloride in standard as well as extracted sample were used in the study.

This shows that, these TLC solvent systems can be used for separation of Dicyclomine Hydrochloride in a mixture of constituents. The method is cheap and easy to perform with all the chemicals and apparatus are readily available in a lab. This methodology of extraction, clean-up and detection can be used as a preliminary, if not confirmatorytesting for Dicyclomine Hydrochloride. Work on the quantitative estimation of analyte extracted from spiked and clinical samples is in progress, and the authors hope to publish the results soon.

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