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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.

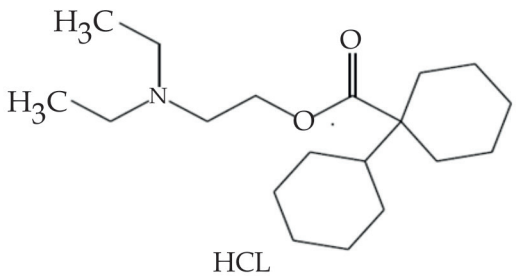

Fig. 1: Chemical structure of Dicyclomine Hydrochloride.

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

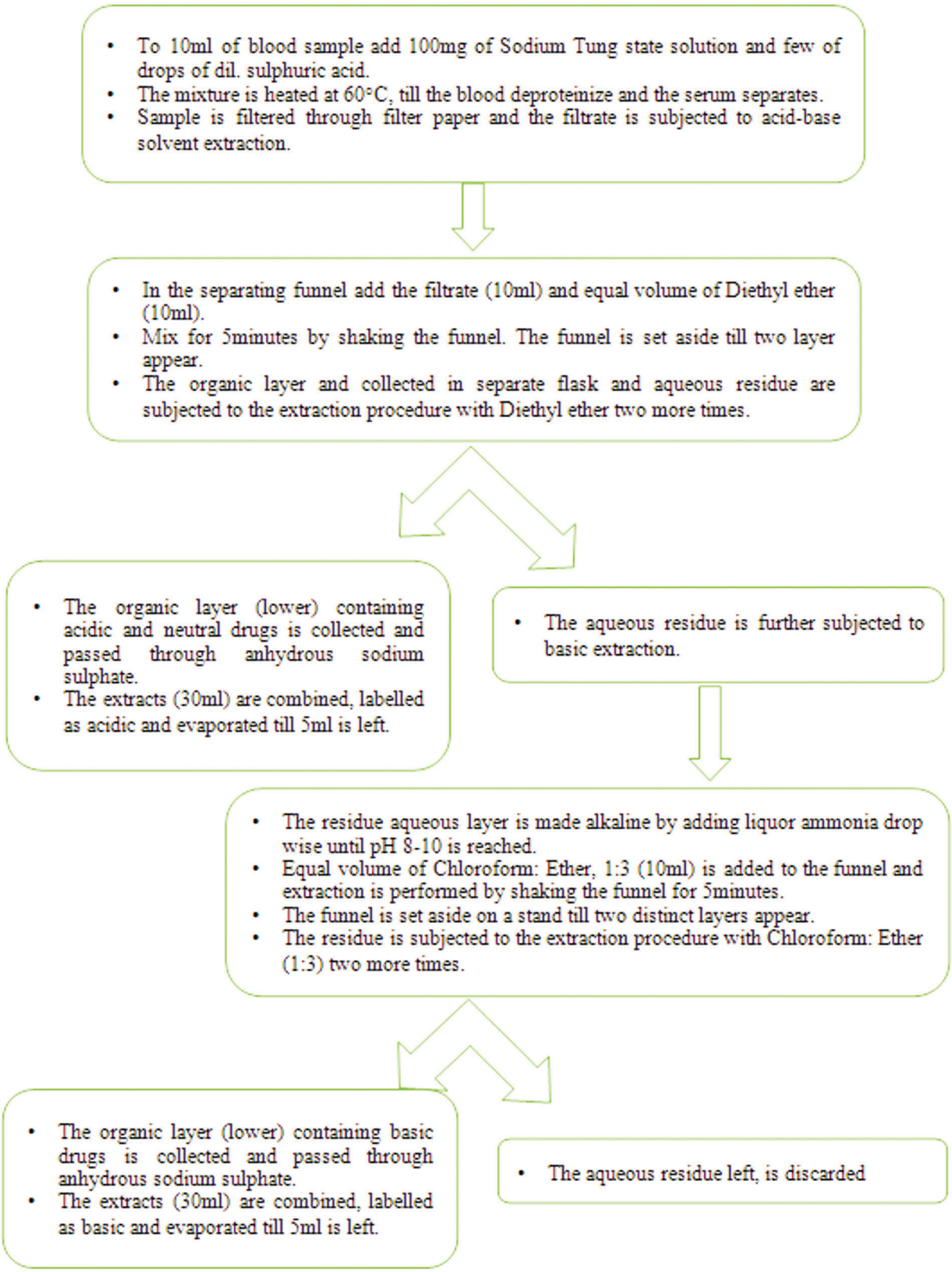

Fig. 2: Schematic Diagram of the Acid-base Extraction Procedure.

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

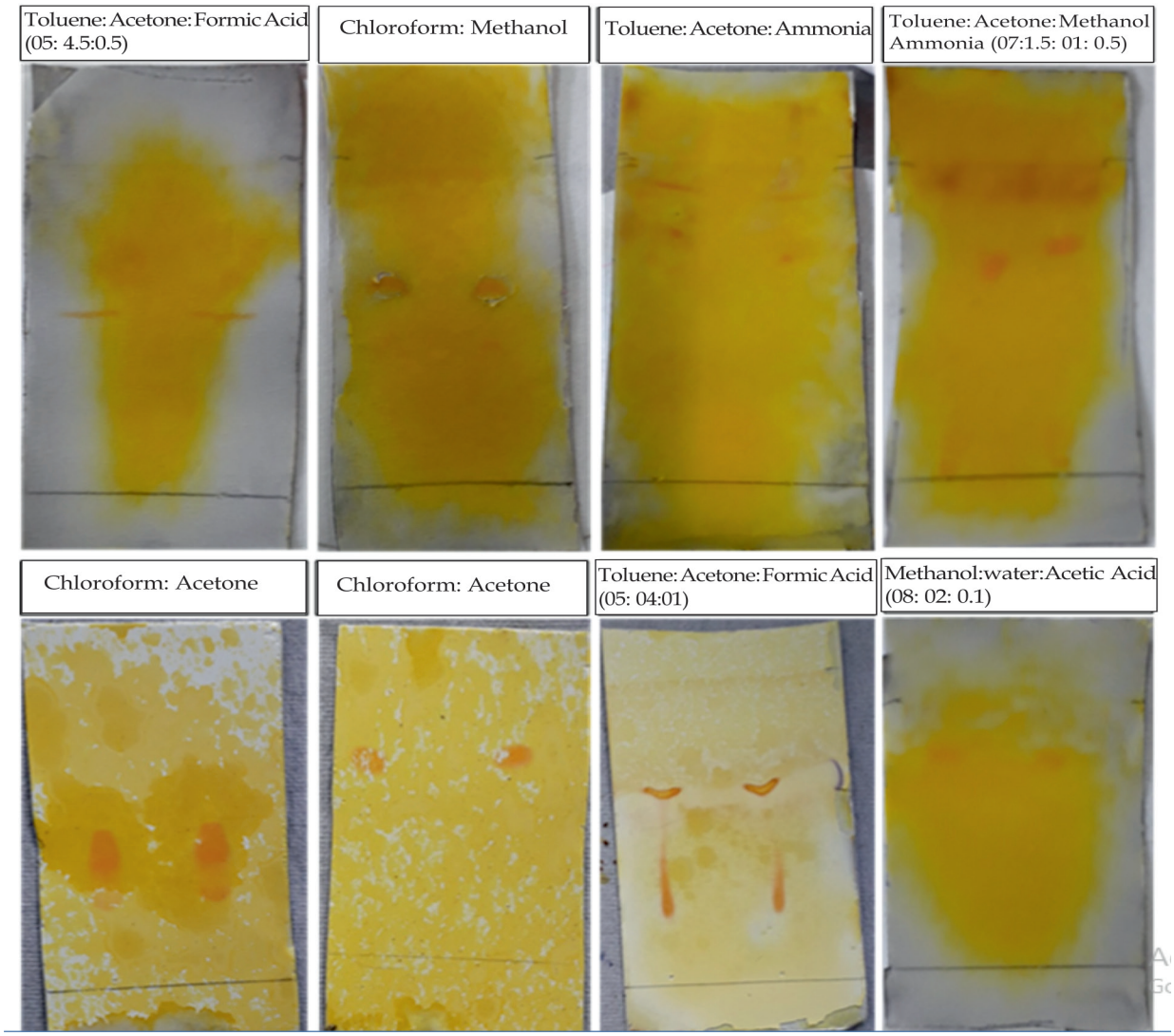

Fig. 3: Results of various mobile phases and visualizing agent (Dragendorff's Reagent).

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.

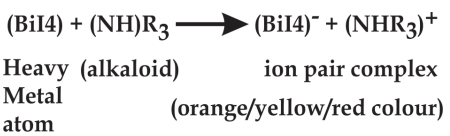

Fig. 4: Reaction of drug with the visualization reagent.

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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Original Article

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Uncertanity Estimation of 28 Pesticides Residues in Chilli by Gas Chromatography, Electron Capture Detector (GC-ECD)

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Abstract

The study was undertaken to calculate the uncertainty of results, related to the analysis of 28 pesticides present in green chilli. Gas Chromatography Electron Capture Detector was used for the analysis of above pesticides present in green chilli. Bottom-up approach was taken for calculation of uncertainty sources arise from weighing, purity of standards, repeatability, calibration and recovery study. To calculate the total uncertainty, relative uncertainty due to purity of standard (U1), due to weighing (U2) and precision (U3) are considered. The combined uncertainty (U) was calculated by equation: $U = [(U1)2+(U2)2+(U3)2]^{1/2}$. Expanded uncertainty (2U) was twice of combined uncertainty (U) at 95% confidence level. Combined uncertainty values lies between 0.0007-0.0035. Percent uncertainty of almost all the pesticides taken for study was found below ≤10% except beta HCH percent uncertainty value is 11% lies in 11-15% range and lambdacyhalothrin value is 16% lies in 15-20% range.

Keywords: Chilli; Uncertanity; Combined Uncertanity; Expanded Uncertanity; Pesticide.

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Introduction

Uncertanity arise either random or as systematic errors which give information about the range of results expected. Uncertanity can be estimated by analytical method of detailed operating procedure. Definition of uncertainty of measurement is a parameter associated with the measurement and dispersion of values attributed to the measurand.[1] The estimation and evaluation of an uncertainty associated-with the result of chemical analysis can be found in SR ENV 13005:2003 guide and in the Eurachem/CITAC Guide CG4. As per SR EN ISO/CEI 17025:2005 all certified laboratories must apply the procedures for the estimation of the uncertainty of the measurement. Bottomup[2], top down and inhouse validation are proposed for expression of uncertainty.[3] Pesticide residue laboratory use bottom-up approach in conjuction with in-house validation[4,5] data for estimating the uncertainty derived from each step of the analytical method.[6-8]

By various analytical steps during the experiment, uncertainty originates from many sources such as sampling matrix effect, uncertainty due to masses, volumetric equipments, reference standards, approximation and assumption are incorporated in the method. Uncertainty of each analytical step consists of its random and systematic error which are qualified and incorporated into the combined standard uncertainty.

This paper based on methodology for estimating the uncertainty associated to multiresidue analytical method in chilli matrix, through the bottom-up

approach and on the basis of in-house validation data.

Materials and Methods

Solvents, Chemicals and Reagents

HPLC grade solvents like. acetone, acetonitrile, ethyl acetate, methanol, and n-hexane were purchased from Merck Germany. Florisil, anhydrous sodium sulphate, sodium chloride, glass wool, celite 545, charcoal, magnesium oxide, cotton, filter paper, and magnesium sulphate anhydrous were purchased from Merck Germany. Primary Secondary Amine i.e. PSA (40 µm, Bondesil) sorbent was purchased from Agilent Technologies. C-18 silica sorbent used in this study was of Supelco and procured from Sigma Aldrich. The use of high purity reagents and solvents help to minimize interference problems. Chilli fruit free of pesticides were obtained from organic farms of Satna district of Madhya Pradesh, India.

Standard Preparation

Pesticide standards were of high purity above 98% were procured from Sigma Aldrich. 28 pesticides under study were (alpha-HCH, beta-HCH, gamma HCH, delta HCH, Alachlor, Aldrin, Dicofol, Pendimethlin, o,p DDE, alpha-Endosulphan, Heptachlor, p,p DDE, Endosulphan Sulphate, Dialdrin, o,p DDD, beta-Endosulphan, p,p DDD, o,p DDT, p,p DDT, Bifenthrin, Fenpropathrin, Lambda Cyhalothrin, beta Cyfluthrin, Cypermethrin, Fenvalarate, Fluvalinate and Deltamethrin). These pesticides are commonly used by farmers of India. CRM of individual pesticide was weighed directly in volumetric flask of 10 ml. on analytical balance (Mettler, Toledo) and dissolved in few drops of HPLC grade acetone which was further reconstituted with HPLC grade n-hexane. Secondary Standard solutions were prepared at, 0.005, 0.01, 0.05, 0.10, 0.50, 1.00 mg/kg which gives good resposnse for Electron Capture Detector of Gas Chromatography. All these working standard solutions of a mixture of pesticides were prepared for calibration and recovery tests.

Extraction and Clean up

QuEChERS (quick, easy, cheap, effective, rugged and safe) method[9] was used for extraction of chilli sample with some modifications. The steps involved are: chilli fruit was finely chopped and homogenized in a mixer grinder. Fifteen gram of homogenized sample weighed into a 50 ml centrifugation tube and 30 ml of Ethyl acetate was added and shaken for 1 min. Ten gram anhydrous Na_2SO_4 was added and shaken. The tube was centrifuged at 6,000 rpm at about 5 minutes. Cleanup was performed according to Lehotay (2007).[10] 6 ml extract was transferred from the upper layer into a 15 ml centrifuge tube, and 0.9 g anhydrous $MgSO_4$, 0.25 g PSA and 0.25 g activated charcoal to remove pigments were added and shaken vigorously for 1 min by vortex shaker. The tubes were centrifuged at 6,000 rpm for 5 min. The supernatant 4 ml was dried in turbovap. The dried sample was reconstituted by adding 1 ml n-hexane. The reconstituted sample was used for GC analysis.

Gas Chromatography – Electron Capture Detector (GC-ECD)

Agilent 7890B (7693 auto sampler) equipped with DB-5MS capillary column (30 meter × 0.25 mm, film thickness 0.25µm) fused silica capillary column was used for preliminary screening and final quantification of pesticide residues. Oven temperature programming was 170°C as initial temperature for 5 min followed by a ramp rate of 2°C/min up to 210°C for 5 min., 1°C/min up to 215°C for 5 min. and 4°C/min. up to final temperature of 280°C with a hold time of 8 min. The injector (splitless mode) and detector temperature were set at 250°C, 300°C, respectively. Injection volume 1.0 micro litre, makeup flow 25ml/min., septum purge flow 3 ml/min. and equilibrium time 1 min. Total flow 63.75 ml/min. with average velocity 18.725 cm/sec and pressure 6.582 psi. Nitrogen was used as makeup gas and helium as carrier gas at a flow rate of 0.75 mL/min.

Determination of Uncertanities

Theoretical aspects of uncertainty estimation

Expressing uncertainty in way different way, standard uncertainty (u(x)), expressed as a standard deviation, and expanded uncertainty (U(x)) which is calculated from a combined standard uncertainty and a coverage factor k. In some cases, it is feasible to use relative uncertainties (in both uncertainties), which represent the value of the uncertainty normalised. It is obtained as the quotient between the standard uncertainty u(x) and the value of x:

$Urel(x) = U(x)/x$

or $urel(x) = u(x)/x$

The steps involved in uncertainty estimation are as follows.

- To Specify the measurand.

- Relationship between the measurand and the input quantities, such as measured quantities, constants and calibration standard values.
- Identify uncertainty sources. Specified uncertainty sources in the above step.
- Quantify uncertainty components. Associated with each potential source of uncertainty identified.
  - The different contributions to the overall uncertainty can be calculated depending on the data available:
  - from a standard deviation value: this value is directly used;
  - from the standard deviation of experimental data sets;
  - from a declared uncertainty value, which is given in a certificate of calibration;
  - from a confidence interval;
  - from a range of limits(upper and lower limits);
  - finally from a given error value.
- Calculate combined uncertainty. The different contributions to the overall uncertainty have to be combined according to the appropriate rules for giving a combined standard uncertainty:

u = square root of ((x* x) + (y* y)+----)

Applying the appropriate coverage factor, the expanded uncertainty will be obtained.

Determination of Uncertanities During Validation of Quantitative chromatography

Method

The measurement uncertainty was calculated as per EURACHEM/CITAC and quantifying uncertainty for 28 pesticides residue in chilli. Uncertanities arise during the experiment are as follows:

1. Standard solution preparation

1.1 Purity of standards

1.2 Weight of standards

1.3 Volumetric flask volume measurement.

1.4 Volume measurement using micropipette

2. Calibration curve preparation

3. Sample Preparation

3.1 Weighing balance

3.2 Volume

4. Repeatability

5. Bias (Recovery)

6. Uncertainty in CRM purity

7. Uncertainty in preparation of std. solution

8. Uncertainty in GC response

Results and Discussions

Uncertainty arise during method validation and analysis of 28 pesticides residues in chilli. The aim of this study was to estimate uncertainties involved in analysis of 28 pesticides residues in chilli involved following steps:

1. identification of of uncertainty sources.
2. quantification of uncertainty sources.
3. calculation of the combined standard uncertainty.

The uncertainty of each individual analytical step consists of its random and systematic component which of these was quantified and incorporated in the combined standard uncertainty. There are many potential sources of uncertainty described in multi-residue methods includes all gravimetric and volumetric steps (sample weighing, dilution of sample extracts, uncertainty of volume of GPC loop, evaporation of sample extracts, temperature, etc.) which contribute to the overall uncertainty. However, detailed exploration and evaluation of all these uncertainty sources is complicated and impractical.

Therefore it is important to evaluate uncertainties of three basic analytical steps. First relative standard uncertainty (U1) due to purity of analytical standards, Uncertainty due to weighing (U2) of analytical CRM, Uncertainty associated with precision (U3) i.e repeatability. Uncertainty is important step for method development process. Combined uncertainty (U) was determined at 0.05 mg/kg level for all the pesticides taken under study as per the statistical procedure of the EURACHEM/ CITAC Guide CG 4[1].

- Identification of Uncertainty Sources
- Repeatability
- Recovery
- Uncertainty in CRM purity
- Uncertanity in weighing
- Uncertainty in preparation of std. solution
- Uncertainty in GC response

- Uncertainty in sample homogeneity

Measur and

ppm conc. = area of sample X conc. of standard X dilution X 1 area of standard sample weight

Quantification of Uncertainty Sources

a. Volumetric flask (10ml). Calibrated, class A glasswares were used, so uncertainty due to glasswares can be neglected.
b. Micro pipette; calibrated pipettes of 1000 and 200 micro litre were used, so uncertainty due to micro pipette can be neglected.
c. GC response; Uncertainty in linearity of response is in given concentration range has been included in the precision study hence separate calculation is not necessary.
d. Sample homogeneity; it can be assumed that pesticide residues are uniformly distributed in the sample. Hence the uncertainty due to sample homogeneity can be ignored.

Main cause of uncertanity

1. First relative standard uncertainty (U1) due to purity of analytical standards.
2. Uncertainty due to weighing (U2) of analytical CRM.
3. Uncertainty associated with precision (U3) i.e repeatability.

Uncertainty by purity of analytical standards (U1)

From all 28 pesticides with their specific purity percent have uncertainty mentioned in the certificate of purity. Rectangular distribution was considered as purity certificate which indicates lack of any confidence level. So by formula, first standard uncertainty SU1 is-. SU1 = (u (x) /√3) where u(x) is the uncertainty value given in the certificate for purity of CRM, and due to rectangular distribution, uncertainty is divided by √3. From uncertainty table 1, uncertainty of all pesticides CRM purity are almost same i.e 0.5% which is converted to (0.005). Whereas relative standard uncertainty (U1) derived according to the equation:. U1 = (SU1 × 100)/% purity. From table 1, the values of relative standard uncertainty were found close to standard uncertainty.

Table 1: Shows the uncertainty calculation due to purity of certified reference standards.

S.No.	Pesticide Standard	Purity of Standard	Uncertanity of Standard (0.05%)	Standard Uncertanity (SU1)	Relative Standard Uncertanity (U1)
1	Alpha-HCH	99.6	0.005	0.0028868	0.0028983
2	Dicofol	99.5	0.005	0.0028868	0.0029013
3	Beta-HCH	99.8	0.005	0.0028868	0.0028925
4	Gamma HCH	99.6	0.005	0.0028868	0.0028983
5	Delta HCH	99.7	0.005	0.0028868	0.0028954
6	Heptachlor	98.2	0.005	0.0028868	0.0029397
7	Alachlor	99.8	0.005	0.0028868	0.0028925
8	Aldrin	99.2	0.005	0.0028868	0.0029100
9	Pendimethlin	99.8	0.005	0.0028868	0.0028925
10	O,P DDE	99.4	0.005	0.0028868	0.0029042
11	Alpha-Endosulphan	99.5	0.005	0.0028868	0.0029013
12	Butachlor	99.3	0.005	0.0028868	0.0029071
13	Dialdrin	99	0.005	0.0028868	0.0029159
14	P,P DDE	99.4	0.005	0.0028868	0.0029042
15	O,P DDD	99.7	0.005	0.0028868	0.0028954
16	P,P DDT	96	0.005	0.0028868	0.003007
17	Beta- Endosulphan	99.5	0.005	0.0028868	0.0029013
18	P,P DDD	96	0.005	0.0028868	0.003007
19	O,P DDT	99.6	0.005	0.0028868	0.0028983
20	Endosulphan Sulphate	99	0.005	0.0028868	0.0029159
21	Bifenthrin	99.5	0.005	0.0028868	0.0029013
22	Fenpropathrin	99.5	0.005	0.0028868	0.0029013
23	Lambda Cyhalothrin	98.5	0.005	0.0028868	0.0029307
24	Beta Cyfluthrin	99.5	0.005	0.0028868	0.0029013
25	Cypermethrin	99.5	0.005	0.0028868	0.0029013
26	Fenvalarate	99.3	0.005	0.0028868	0.0029071
27	Fluvalinate	99.8	0.005	0.0028868	0.0028925
28	Deltamethrin	99.5	0.005	0.0028868	0.0029013

Uncertainty of weighing (U2)

The uncertainty arise during weighing of neat standards. Weight of standards were taken between 1-2 mg. The uncertainty value of the weighing balane is 0.001gm. The normal distribution of weight is taken under consideration. Standard uncertainty due to weighing calculated by the equation = 0.0001/2, whereas relative standard uncertainty U2=(0.0001/2)/W, whereas W is the weight of pest the pesticide standard weighed using precision analytical balance, 0.0001 is the value of uncertainty at 95% confidence level taken from the valid calibration certificate of balance. Considering normal distribution, the uncertainty of the balance was divided by taking two. The calculation of uncertainty due to weighing of certified reference standards are shown in Tabe 2.

Uncertainty arise due to precision (U3)

Uncertainty arise due to precision are shown in table 3. Table shows that for test mixture of 28 mixture pesticides, three replicate recovery and their mean value, standard deviation, relative standard deviation were calculated. , Errors caused during sample processing steps i.e extraction, clean up, and GC analyses were approximated by standard deviations (s), calculated from triplicate determinations of analytes expressed as repeatability by equation: U3 = s/(√n × x) where standard deviation (s) is obtained from the recovery study, n is the number of replications and x is the mean value of the concentration recovered.

Uncertanity Budget

To calculate the total uncertainty, Relative uncertainty due to purity of standard (U1), due to weighing (U2) and precision (U3) are considered. For calculating combined uncertainty, the sum of the square root of U1, U2 and U3 are taken. The combined uncertainty (U) was calculated by equation: U = x [(U1)2 + (U2)2 + (U3)2]1/2. Expanded uncertainty (2U) was twice of combined uncertainty (U) at 95% confidence level. From table no.4, combined uncertainty values lies between 0.0007-0.0035. Also percent uncertainty value is calculated by dividing expanded uncertanity value by recovered amount value and multiplied by 100. From the table 4. The expanded uncertainty of the

Table 2: Shows the uncertainty calculation due to weighing of certified reference standards.

S.No.	Pesticide Standard	Weight of Standard	Uncertanity in Weighing	Standard Uncertanity	Relative Standard Uncertanity (U2)
1	Alpha-HCH	1.24	0.0001	5.77E-05	4.66E-05
2	Dicofol	1.91	0.0001	5.77E-05	3.02E-05
3	Beta-HCH	1.37	0.0001	5.77E-05	4.21E-05
4	Gamma HCH	1.86	0.0001	5.77E-05	3.10E-05
5	Delta HCH	1.48	0.0001	5.77E-05	3.90E-05
6	Heptachlor	1.2	0.0001	5.77E-05	4.81E-05
7	Alachlor	1.25	0.0001	5.77E-05	4.62E-05
8	Aldrin	1.23	0.0001	5.77E-05	4.69E-05
9	Pendimethlin	1.73	0.0001	5.77E-05	3.34E-05
10	O,P DDE	1.8	0.0001	5.77E-05	3.21E-05
11	Alpha-Endosulphan	1.45	0.0001	5.77E-05	3.98E-05
12	Butachlor	1.27	0.0001	5.77E-05	4.55E-05
13	Dialdrin	1.56	0.0001	5.77E-05	3.70E-05
14	P,P DDE	1.84	0.0001	5.77E-05	3.14E-05
15	O,P DDD	1.87	0.0001	5.77E-05	3.09E-05
16	P,P DDT	1.82	0.0001	5.77E-05	3.17E-05
17	Beta- Endosulphan	1.57	0.0001	5.77E-05	3.68E-05
18	P,P DDD	1.46	0.0001	5.77E-05	3.95E-05
19	O,P DDT	1.83	0.0001	5.77E-05	3.15E-05
20	Endosulphan Sulphate	1.74	0.0001	5.77E-05	3.32E-05
21	Bifenthrin	1.46	0.0001	5.77E-05	3.95E-05
22	Fenpropathrin	2.1	0.0001	5.77E-05	2.75E-05
23	Lambda Cyhalothrin	1.54	0.0001	5.77E-05	3.75E-05
24	Beta Cyfluthrin	1.36	0.0001	5.77E-05	4.25E-05
25	Cypermethrin	1.45	0.0001	5.77E-05	3.98E-05
26	Fenvalarate	1.89	0.0001	5.77E-05	3.05E-05
27	Fluvalinate	1.87	0.0001	5.77E-05	3.09E-05
28	Deltamethrin	1.56	0.0001	5.77E-05	3.70E-05

Table 3: Shows Recovery, Mean Recovery, Standard Deviation (S.D), Relative Standard Deviation (RSD) of organochlorine, synthetic pyrithroids and herbicides pesticides from spiked chilli matrix at 0.05 ppm.

S. No.	PESTICIDE	RT	Spiking conc (PPM)	Amount recovered R1	Amount recovered R2	Amount recovered R3	Mean Rec. Amount	Standard Deviation	Standard Uncertainty	Relative Standard Uncertainty (U3)
1	Alpha-HCH	9.8	0.05	0.041	0.043	0.046	0.043	0.0025	0.0015	0.03379
2	Dicofol	10.8	0.05	0.046	0.043	0.042	0.044	0.0021	0.0012	0.027315
3	Beta-HCH	11.25	0.05	0.041	0.049	0.048	0.046	0.0044	0.0025	0.054709
4	Gamma HCH	11.57	0.05	0.046	0.04	0.041	0.042	0.0032	0.0019	0.044189
5	Delta HCH	12.86	0.05	0.041	0.046	0.045	0.044	0.0026	0.0015	0.034716
6	Heptachlor	15.78	0.05	0.046	0.043	0.045	0.045	0.0015	0.0009	0.019598
7	Alachlor	15.87	0.05	0.045	0.043	0.042	0.043	0.0015	0.0009	0.02051
8	Aldrin	18.05	0.05	0.044	0.04	0.046	0.043	0.0031	0.0018	0.041019
9	Pendimethlin	21.06	0.05	0.045	0.042	0.043	0.043	0.0015	0.0009	0.02051
10	O,P DDE	23.21	0.05	0.045	0.042	0.045	0.044	0.0017	0.0010	0.022727
11	Alpha-Endosulphan	23.52	0.05	0.044	0.042	0.043	0.043	0.0010	0.0006	0.013427
12	Butachlor	24.22	0.05	0.045	0.044	0.043	0.044	0.0010	0.0006	0.013122
13	Dialdrin	25.53	0.05	0.041	0.042	0.046	0.043	0.0026	0.0015	0.035524
14	P,P DDE	25.68	0.05	0.045	0.042	0.039	0.042	0.0030	0.0017	0.041239
15	O,P DDD	26.3	0.05	0.045	0.039	0.043	0.042	0.0031	0.0018	0.041996
16	P,P DDT	26.4	0.05	0.039	0.042	0.043	0.041	0.0021	0.0012	0.029313
17	Beta- Endosulphan	28.14	0.05	0.043	0.041	0.044	0.043	0.0015	0.0009	0.02051
18	P,P DDD	29.47	0.05	0.04	0.042	0.042	0.041	0.0012	0.0007	0.01626
19	O,P DDT	29.72	0.05	0.046	0.042	0.041	0.043	0.0026	0.0015	0.035524
20	Endosulphan Sulphate	32.6	0.05	0.044	0.043	0.046	0.044	0.0015	0.0009	0.020044
21	Bifenthrin	41.72	0.05	0.045	0.043	0.046	0.045	0.0015	0.0009	0.019598
22	Fenpropathrin	42.19	0.05	0.045	0.043	0.046	0.045	0.0015	0.0009	0.019598
23	Lambda Cyhalothrin	47.44	0.05	0.042	0.043	0.042	0.042	0.0006	0.0003	0.007937
24	Beta Cyfluthrin	52.7-52.9	0.05	0.044	0.046	0.043	0.044	0.0015	0.0009	0.020044
25	Cypermethrin	53.03-53.44	0.05	0.046	0.041	0.042	0.043	0.0026	0.0015	0.035524
26	Fenvalarate	56.23	0.05	0.044	0.043	0.046	0.044	0.0015	0.0009	0.020044
27	Fluvalinate	56.9-57.2	0.05	0.043	0.045	0.046	0.045	0.0015	0.0009	0.019598
28	Deltamethrin	58.63	0.05	0.046	0.041	0.043	0.043	0.0025	0.0015	0.03379

Table 4: Results of individual and combined uncertainties with expanded uncertainty for of organochlorine, synthetic pyrithroids and herbicides pesticides from chilli matrix at 0.05 ppm.

S. No.	Pesticide	Mean Recovered	U1	U2	U3	U	2U	Uncertainty	Percent Uncertainty
1	Alpha-HCH	0.043	0.0028983	4.66E-05	0.03379	0.0014	0.0029	±0.003 of 0.043	7
2	Dicofol	0.044	0.0029013	3.02E-05	0.027315	0.0012	0.0024	±0.002 of 0.044	5
3	Beta-HCH	0.046	0.0028925	4.21E-05	0.054709	0.0025	0.0051	±0.005 of 0.046	11
4	Gamma HCH	0.042	0.0028983	3.10E-05	0.044189	0.0019	0.0037	±0.004 of 0.042	9
5	Delta HCH	0.044	0.0028954	3.90E-05	0.034716	0.0015	0.0030	±0.003 of 0.044	7
6	Heptachlor	0.045	0.0029397	4.81E-05	0.019598	0.0009	0.0018	±0.002 of 0.045	4
7	Alachlor	0.043	0.0028925	4.62E-05	0.02051	0.0009	0.0017	±0.002 of 0.043	4
8	Aldrin	0.043	0.00291	4.69E-05	0.041019	0.0018	0.0036	±0.004 of 0.043	8
9	Pendimethlin	0.043	0.0028925	3.34E-05	0.02051	0.0009	0.0017	±0.002 of 0.043	4
10	O,P DDE	0.044	0.0029042	3.21E-05	0.022727	0.0010	0.0020	±0.002 of 0.044	5
11	Alpha-Endosulphan	0.043	0.0029013	3.98E-05	0.013427	0.0006	0.0012	±0.002 of 0.043	3
12	Butachlor	0.044	0.0029071	4.55E-05	0.013122	0.0006	0.0012	±0.002 of 0.044	3
13	Dialdrin	0.043	0.0029159	3.70E-05	0.035524	0.0015	0.0030	±0.003 of 0.043	7
14	P,P DDE	0.042	0.0029042	3.14E-05	0.041239	0.0017	0.0035	±0.004 of 0.042	8
15	O,P DDD	0.042	0.0028954	3.09E-05	0.041996	0.0018	0.0036	±0.004 of 0.042	9
16	P,P DDT	0.041	0.003007	3.17E-05	0.029313	0.0012	0.0024	0.003 of 0.041	6
17	Beta- Endosulphan	0.043	0.0029013	3.68E-05	0.02051	0.0009	0.0017	0.002 of 0.043	4
18	P,P DDD	0.041	0.003007	3.95E-05	0.01626	0.0007	0.0014	0.002 of 0.041	3
19	O,P DDT	0.043	0.0028983	3.15E-05	0.035524	0.0015	0.0030	0.003 of 0.043	7
20	Endosulphan Sulphate	0.044	0.0029159	3.32E-05	0.020044	0.0009	0.0018	0.002 of 0.044	4
21	Bifenthrin	0.045	0.0029013	3.95E-05	0.019598	0.0009	0.0018	0.002 of 0.045	4
22	Fenpropathrin	0.045	0.0029013	2.75E-05	0.019598	0.0009	0.0018	0.002 of 0.045	4
23	Lambda Cyhalothrin	0.042	0.0029307	3.75E-05	0.007937	0.0035	0.0069	0.007 of 0.042	16
24	Beta Cyfluthrin	0.044	0.0029013	4.25E-05	0.020044	0.0009	0.0018	0.002 of 0.044	4
25	Cypermethrin	0.043	0.0029013	3.98E-05	0.035524	0.0015	0.0030	0.003 of 0.043	7
26	Fenvalarate	0.044	0.0029071	3.05E-05	0.020044	0.0009	0.0018	0.002 of 0.044	4
27	Fluvalinate	0.045	0.0028925	3.09E-05	0.019598	0.0009	0.0018	0.002 of 0.045	4
28	Deltamethrin	0.043	0.0029013	3.70E-05	0.03379	0.0014	0.0029	0.003 of 0.043	7

U1 = Relative Standard Uncertainity of analytical standards; U2 = Relative Standard Uncertainity of weighing; U3 = Uncertainity associated with precision; U = Combined Uncertainity; 2U = Expanded Uncertainity

pesticides was under three ranges viz., (a) ≤10% (b) 11–15% and (c) 15–20%. Percent uncertainty of almost all the pesticides taken for study was found below ≤10% lies (a) range except beta HCH percent uncertainty value is 11% lies in (b) range and lambdacyhalothrin value is 16% lies (c) range. Table 4. shows individual uncertanities and combined uncertainties with expanded uncertainty for 28 pesticides from spiked chilli matrix at 0.05 ppm.

Conclusion

The method followed for all pesticides taken for study is efficient in determining of uncertainty of 28 pesticides from chilli matrix. Uncertanity value is calculated for each major step of method validation. Uncertanity arise by various steps of the method are rectified and calculated according to SANCO guidelines.

Aknowlegement

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Molecular Aspect of Post-mortem interval (PMI)

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Abstract

Post-mortem interval (PMI) is a major component of investigation in forensic science. It is one of the fundamental tasks for forensic pathologist when a body is found. According to criminal law point of view, a correct estimation of the PMI allows authentication of witness statements, thus restrict the number of suspects. Accurate PMI estimation is difficult because of intrinsic and extrinsic factors. Analysis of the biochemical changes in glucose and electrolytes for PMI estimation has made significant progress. Molecular ways in which measurable or quantifiable technique for degradation of nucleic acid molecule such as DNA and RNA, may be the good indicator for PMI. Several studies have tried to determine changes in molecular markers to provide more useful information for PMI. The studies demonstrated mixed results that showed the influence of ante and post-mortem factors on nucleic acid degradation. More studies would be required in order to standardize on human cases under standard parameters. Thus, molecular aspects of forensic involves this application of omicsin medical sciences to investigate cause of death and its process at the genetic basis and biological molecular level.

Keywords: Post-mortem interval (PMI); Molecular biology; DNA and RNA quantification; Forensic Genetic identification**.**

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Introduction

Estimation of Post-mortem interval (PMI) or time since death is vital tool for crime Investigation and homicide victim in the field of forensic medicine. Post mortem interval is the period between death and actual performance of autopsy/post-mortem examination. It is also one of the fundamental tasks of the forensic pathologist to determine PMI in Medico-legal autopsies.[1] From the criminal law point of view, correct analysis of the PMI permits endorsement of witness statements, thus restricts the number of suspects. There are various methods used to estimate PMI such as corporal evidence-that which are present in dead body, physical methods based on measurement of body temperature, the electrical stimulation of facial muscles, environmental and associated evidence –that which is present in the vicinity and general surrounding of deceased and anamnestic evidence-that which is based on the deceased ordinary habits, movements and day to day activity.[2,3] It is important to calculate time of death with accuracy and correctness. These methods can be included into routine death investigations and training programs in forensic for assessment of cause and process of death according to autopsy and laboratory findings. This article is mainly focusing on molecular approaches towards the PMI.

Molecular Role of DNA and RNA degradation

It has been reported that three main molecular ways in which the nucleic acid molecule (DNA and RNA) degrades in a living person during hydrolysis, oxidation, and methylation where natural repair processes exist in the body to handle all these mechanisms. In contrast to this degradation of

DNA and RNA begins directly after death, when the body's natural repair mechanisms of DNA and RNA are halted. The breakdown process is influenced by several environmental factors, including heat, light, and humidity; this natural variation is greater than that of the degradation between individual samples.[4]

During post-mortem autolysis, cellular organelles and nuclear acid break down into their constituent parts. If degradation occurs at a particular amount; can this amount be determined using laboratory methods? Theoretically, this information may be used to estimate PMI for an individual who has been dead a number of days or hours by several workers. After death the stability of DNA is variable in different body organs5. According to Bär et al. (1988:68-69), the good stability of DNA occurs in the "brain cortex, lymph nodes and psoas muscle after the 3 weeks of time. Trotter SA and workers in 2002 examined the effect of post-mortem interval (PMI) on mRNA by utilizing gene arrays. Their studies were conducted on mice which were similar circumstances of post mortem collections of human brains. These studies reported that after 8–24 h gene expression correlation and equivalency not match, but 90–95% of detected genes were within ±40% of baseline levels. Brain homogenate pH did not change with PMI and suggested such studies should be carried out to determine the effect of post-mortem interval on larger gene population distributions.[6] This type of data would appear essential to interpreting gene array studies of human brain diseases that utilize post-mortem brains and should be validated for the arrays used in any particular study.

Dong Zhao et.al 2006 have conducted the study to determine quantitative test of oxygen-regulating factors with erythropoietin (EPO), vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1 alpha (HIF1A) mRNAs, and to examine the post-mortem stability of those mRNA transcripts in forensic autopsy samples. Relative quantification of EPO, VEGF and HIF1A mRNAs, based on the TaqMan reverse transcription-polymerase chain reaction (RT-PCR), was performed on autopsy tissue samples from the heart (nZ10), brain (nZ10), kidney (nZ16) and lung (nZ8) after preservation at room temperature for various storage times. VEGF and HIF1A mRNA gradually degraded in patterns related to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA used as an endogenous reference. They reported that the relative quantification of VEGF/GAPDH and HIF1A/GAPDH slightly differ to 48 h post-mortem in tissue samples in comparison of the brain, kidney and lung with no deviation of HIF1A in the myocardium. However, the condition was different for EPO mRNA, with exaptational stability for post-mortem degeneration and a distinct post-mortem time-dependent increase in the EPO/GAPDH ratio for all tissue samples7,8. Their study has suggested the potential for applying quantitative analyses of mRNA transcripts at autopsy samples indicated the understanding of study degradation profiles before to accomplish relative quantification of target mRNAs in autopsy samples.

ChengzhiLia, b et. al 2016 reviewed the developments in the evaluation of PMI by forensic researchers from china (1984 to 2015) using degradation of DNA, RNA, entomological methods, spectroscopic technology, energy changes, estimation of energy changes in the body after death (cooling or blood ATP levels), thanato chemistry (chemistry of death by describing changes in the chemical composition of various body fluids); and other methods activity imaging technology, electrophysiological methods and enzyme.[9] Elghamry et. al 2017 studied Post-mortem degradation of RNA for PMI estimation to understand association between the quantity of remaining RNA and the proceeding time. They used GAPDH mRNA quantity in the brain as a indicator for PMI in different environmental conditions, they reported GAPDH mRNA in rat's brain could be a helpful marker for PMI estimation in various environmental conditions10.Jianlong Ma et.al and Ye-Hui Lv et.al 2017 are same group of workers examined various RNA markers in human and rat tissues, they were screened valid biomarkers and also established and validated corresponding mathematical models to determine precise estimation of PMI with R software which may simultaneously manage both PMI and temperature parameters. Similarly, multi-RNA markers of myocardium and liver tissues were detected by quantitative PCR and reported the Ct values of ten markers with increased duration of PMIs. Among all 5 S, miR-1 and miR-133a were shown to be optimum reference biomarkers that were not affected by a PMI of up to 5 or more days; whereas liver-specific miR-122 started to degrade under higher temperatures and only 5S was selected as an endogenous control in the liver. Among the tested target RNAs, they reported that their previous study in brain tissue, β-actin (ΔCt) was found to exhibit the best correlation coefficient with PMI and was employed to build mathematical models using R software. In terms of validation, the comparably low estimated error demonstrated

that PMIs can be accurately predicted in human cases through complete consideration of numerous factors and using effective biomarkers.[11,12] In another study conducted on mRNA marker to assess PMI with accuracy. A hypoxia associated factor (HAF) mRNA degradation within48 h after death were performed to seek precise time of PMI determination in mouse brain, for which relative quantitative PCR was used to observe expression level of HAF mRNA and Caspas-3DNA where Caspase- 3 DNA was performed as normalized HAF mRNA degradation.The profiling of HAF mRNA degradation were analysed through a statistical model between 48 h PMI and mRNA degradation. They found 105 bp HAF mRNA increased fragment in 48 h that suggested PMI was well correlated with HAF mRNA degradation in mouse brain. These results indicated, HAF mRNA was a suitable marker for PMI determination and the statistical model is a useful tool in forensic practice for time since death.[13]

Birds ill AC et al 2011 isolated and analysed RNA of brain samples from the Banner Sun Health Research Institute Brain and Body Donation Program in order to determine the relationship between PMI and RNA integrity. A PCR- based gene expression array was used to understand how can PMI affects the expression of large set of gene(n=89). In this study they correlated the PMI ranged from 1.5 to 45hours with total RNA quality measures including RNA integrity and RNA quantity yield. The results demonstrated that greater proportion of genes had decreased expression instead of increased with increasing PMI (65/89vs 20/89).This study concluded that RNA degrades continuously on increasing PMI and that measurement of gene expression in brain tissue with longer PMI may give pretended low values. For tissue derived from autopsy, a short PMI optimizes its utility for molecular research.[14] Based on DNA quantification studies Mansour H et.al in 2019,evaluated outcome of antemortem and post-mortem aspects on dental DNA in actual forensic cases.The outcome of antemortem and post-mortem aspects on dental DNA in actual forensic cases. 95 teeth were extracted from 39 corpses, that were subjected to 6 different post-mortem conditions. A real-time PCR technique were used to measures DNA concentration to evaluate the PMI. The results showed first ten days of time period yielded best DNA from all analysed dental samples, whereas decreased yield of dental DNA was observed after following time period. They also reported that the amount of DNA from fresh and burnt corpse was high in yield instead of skeletonized exhumed corpse. Those who are dried and indoor condition showed better result infect those were in water, outdoor, buried in ground. In terms of antemortem factors no significant yield of dental DNA were revealed. This study suggested ante mortem factors are more significant to individual variations whereas post-mortem factors have significant influence on dental DNA amount yielded.[15] Stephanie T. et. al have studied in 2013 estimating post-mortem interval by performing technique for RNA degradation and observed morphological changes in tooth pulp in animal.[16]

Conclusion

Accurate PMI estimation is difficult because of intrinsic and extrinsic factors. Analysis of the biochemical changes in glucose and electrolytes for PMI estimation has made significant progress. It is considered that DNA and RNA are the feasible parameter for PMI estimation because they are most stable component of the cells. These molecules are similar among different individuals and different cell types within same genus. It is demonstrated that denaturation begins immediately in biological samplesandcontinues at a constant rate, regardless of the temperature and the mechanisms of death. It is concluded that most of the studies concentrating on animal models on tissues of particular organs and demonstrated mixed results that representing influence of ante and post-mortem factors on nucleic acid degradation. More studies are required in response to standardize on human cases under standard parameters.

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Case Report

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Atypical Firearm Wounds, One Projectile Leading to Multiple Injuries: Correct Interpretation and Correlation

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Abstract

In the practice of Forensic medicine, firearm injuries cases pose a challenge, particularly those caused during the police/armed forces action also known as 'Encounter'. Autopsy reports play a very important role in recreation/reconstruction of the event and are an important evidence for review by other agencies. The track of the wounds, direction of firing, site of entry and exit wounds along with other internal injuries are used to authenticate and verify the version of police officers. Authors have observed that in certain cases of death consequent to firearm injury, sometimes atypical wounds are found which may be difficult to interpret at the time of performing autopsy. A meticulous postmortem examination coupled with cautious analysis and interpretation of injuries has to be done before furnishing a Medico legal opinion in such cases. The authors report such a case in which an atypical firearm wound was found in a police encounter case. The case is being reported only for academic purposes to highlight the importance of correct interpretation in such atypical wounds by a meticulous autopsy examination so as to prevent any further doubts in their correlation with sequence of events. Modern radiological techniques like Digital X-ray and PM MSCT should be used in firearm cases to enhance the objectivity and credibility of postmortem findings.

Keywords: Firearm injuries; Encounter Deaths; Gunshot Wounds; Post Mortem Multi slice Computed Tomography.

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Introduction

In the practice of Forensic medicine, autopsy surgeons frequently come across the cases of death due to firearm injuries in the normal course of duties. Many of such cases pose a challenge, particularly those caused during the police/armed forces action also known as 'Encounter'. These encounter cases are always disputed by the relatives of the persons killed in the police action.[1,2,3] These cases may be subjected to reinvestigation/review by other agencies like Central Bureau of Investigation, National Investigating Agency, and Honorable Courts of law.[4] Police encounter cases are mandatorily reviewed by National Human Rights Commission (NHRC). NHRC has framed a separate procedure for conducting inquest and performing autopsies in such shoot out cases.[5] Autopsy reports play a very important role in recreation/reconstruction of the event and are an important evidence for review by other agencies. The track of the wounds, direction of firing, site of entry and exit wounds along with other internal injuries are used to authenticate and verify the version of police officers. Authors have observed that in certain cases of death consequent to firearm injury, sometimes atypical wounds are found which may be difficult to interpret at the time of performing autopsy. Identification of the wounds,

track of the bullet and recovery of the bullet may throw serious challenges as sometimes the findings may not match with the version of police personnel. Thus a meticulous postmortem examination coupled with cautious analysis and interpretation of injuries has to be done before furnishing a Medico legal opinion in such cases. The authors report such a case in which an atypical firearm wound was found in a police encounter case. The case is being reported only for academic purposes to highlight the importance of correct interpretation in such atypical wounds by a meticulous autopsy examination so as to prevent any further doubts in their correlation with sequence of events.

Case Report

The limited details of the case will only be shared for maintaining the confidentiality and also as the case may be under judicial proceedings/ enquiry. Minimal relevant Autopsy findings are being mentioned to fulfill the objective of the Academic deliberation and for addition to the Medical Literature. The external findings, clothing examination, other firearm injuries over the body and internal injuries will not being disclosed.

The deceased was a young adult male, who sustained firearm injuries in a Police encounter. He was declared as brought dead in a hospital. Autopsy was conducted under Videography by a Medical Board, as per the established protocol. Deceased was subjected to X-rays examination to check for the presence of any projectile in-situ and the result was negative.

While correlating the wounds based on the presence of features of entry and exit wounds, it was concluded that a single bullet has caused four external injuries which are not lying on the same line when the head is kept in anatomical position (as marked by a red arrow in Fig 1A). Two bullet-grazed reddish abrasions (A&B) are noted which are placed obliquely directed downwards (Fig 1B).

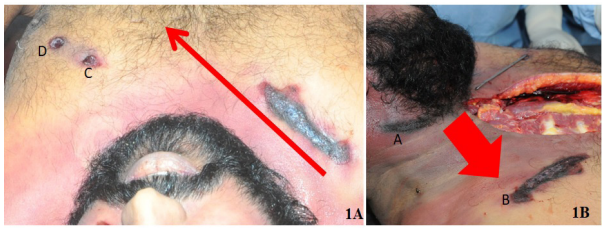

Fig. 1: (A&B).

One is present on the right side of neck involving the mandibular region along the outer aspect of floor of the mouth and the second one is present involving the right infra clavicular region. Both the wounds correspond to each other upon flexing the head towards right side. The wound tract is running obliquely downwards and medially towards left side. An entry wound with abrasion collar towards the right side is present over the inner aspect of the left chest and exiting along the outer and lower aspect of the left chest traversing along the subcutaneous plane. The underlying soft tissues were contused devoid of any rib fractures. The above mentioned four injuries (A, B, C, D) were in a single line when the deceased head was flexed over the right side of chest. Hence it was concluded that the projectile travelled from above downwards at the time of shootout.

Discussion

Atypical firearm wounds have the potential for multiple issues of wrong interpretation. Knights et al had illustrated a case where a there was a single entry wound over the left temple and two exit wounds over the right tempero-parietal region which was caused by a. 22 rifle. The two bullets that had entered the skull through a single-entry wound had diverged inside the skull to exit through the two exit wounds. At times there may be there may not be an exit wound but bullet may be recovered from the clothing.[6]

Atypical entry wounds comprise of the graze and the tangential wounds. The graze wound is produced by the passage of the projectile across the skin surface, causing a superficial wound with little penetration to the skin. The wound appears shallow and elongated with a gutter. In majority of such cases, there is a degree of taper at each end which gives a symmetrical configuration. The presence of peripheral abrasion rims in these wounds is not the same in all cases. A small skin split helps in commenting upon the direction of entry. The depth of the wound varies according to the movement of the projectile and might create confusion in deciding whether it is a first contact or exit.[7]

In tangential firearm wounds the projectile contacts the skin at an angle. They are deeper than the graze wounds produced during the motion of the bullet. These wounds are asymmetrical in appearance and margins are ragged and torn. These tear and split lacerations guide the Medical examiner in concluding the direction of the projectile as in our case. There is extensive tissue loss in case of high velocity tangential wounds.[7] Our case report illustrated atypical entry wound with the presence of graze wounds and tangential wounds with presence of contusion, furrowing,

skin splits and tears. The above-mentioned findings helped us to conclude the relative position of the deceased as mentioned above. It also illustrates that the formation of atypical wound depends on the relative position of the individual.

A single bullet has caused a total of five injuries which helped in correlating the relative position of the victim. The five injuries listed are four external injuries i.e. the two grazed abraded contusion, an entry wound with an exit wound, contusion of the intercostal muscles. In an another case reported by Singh V P et al, a single bullet has caused five wounds based on which they concluded the possible position of the victim.[9] The incidence of such type of cases is prevailing but with a lesser frequency. A study by James et al regarding the Firearm deaths by law enforcement in United States found that almost more than 50% of the deaths involved more than two or more entry wounds.[9] Our case findings were in accordance with James et al. Studies suggest that in cases of involvement of multiple wounds, head is considered to be the most predominant site after chest.[10-12] However this finding of comparative study can't be commented/ cited upon, due to confidentiality issues.

The authors find it pertinent to mention the importance of use value of radiological techniques in such cases. Currently 2-D digital X-ray examination is used to study the wound ballistics and locate the bullets/projectile. In case of X rays more than two views are needed to localize the foreign body. The decomposition changes and maggot infestation can alter the wound morphology of such atypical firearm wound. The usage of Post Mortem Multi slice Computed Tomography (PM MSCT) in Gunshot cases will be better in interpreting the findings as compared to the X rays.[13,14]

Conclusion

These types of cases are a real challenge to the forensic experts on the autopsy table. The key to solve such cases is the correct observations and application of mind to correlate the injuries with the help of the features like abrasion collar, direction of minute skin tags and bullet graze etc. Modern radiological techniques like Digital X-ray and PM MSCT should be used in firearm cases to enhance the objectivity and credibility of postmortem findings. The presence of three dimensionality in PM MSCT makes it possible to exact the location and recovery of projectile and avoids the tedious search of the assumed bullet interpreted in 2-D X rays.

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