Chromatography And Separation Techniques Journal

SCIENTIFIC LITERATURE

Special Issue Article "High-Performance Thin-Layer Chromatography" Research Article

# Chlorpyrifos Acute Fatal Poisoning in Humans: Analysis of Whole Blood Samples by a Validated High-Performance Thin-Layer Chromatography

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**ARTICLE INFO**

Received Date: August 05, 2022
Accepted Date: September 12, 2022
Published Date: September 14, 2022

**KEYWORDS**

Liquid–liquid extraction
Whole blood
Chlorpyrifos
High–performance thin–layer chromatography
Validation



**Citation for this article:** Praveen U Sanganalmath, Purigali M Nagaraju and Kuruba Sreeramulu. Chlorpyrifos Acute Fatal Poisoning in Humans: Analysis of Whole Blood Samples by a Validated High-Performance Thin-Layer Chromatography. Chromatography And Separation Techniques Journal. 2022; 3(2):125

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## ABSTRACT

An optimized extraction procedure followed by a validated High–Performance Thin–Layer Chromatography (HPTLC) was applied for the assay of chlorpyrifos (CPF) levels in postmortem blood obtained in seven fatal intoxication cases. The proposed method involved liquid–liquid extraction of CPF with different solvents over a sample pH 3 to 7 and separation was achieved on precoated silica gel $60F_{254}$ TLC plates by using mobile phase consisted of *n*–hexane–acetone (9:1, *v/v*). Densitometric scan was performed at 295 nm in absorbance mode. Optimum extraction was achieved by using toluene solvent at sample pH 5. Linearity was established in the range 2 to 100µg/mL and sensitivity was 1.97µg/mL. The within-day and between-day accuracy was within 14% and precision was less than 2.57% at the three concentration levels 2, 10 and 50µg/mL. The average recovery of CPF analyzed within-and between-day intervals was 89.39%. No decomposition of CPF was observed in the stability samples. The estimated CPF concentrations in postmortem blood samples were in the range of 5.92 to 31.44µg/mL. The proposed HPTLC method is simple, rapid and sensitive with adequate precision and accuracy, thus allowing the direct application of the method in the forensic toxicological analysis.

## INTRODUCTION

Chlorpyrifos, CPF (O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate) is a commonly used pesticide belongs to heterocyclic organothiophosphate group and widely applied in agriculture and urban pest control. CPF containing pesticide formulations are registered in India for control of pests on various crops, fruits and vegetables [1]. Pesticide formulations containing CPF as a single active substance are available in market as 20% Emulsifiable Concentrate (EC), 50% EC, 10% Granules (G), 1.5% Dustable Powder (DP), 2% Ready-To-Use (RTU), 20% Capsule Suspension (CS) or in combination of CPF 20% EC + acetamiprid 0.4% EC, CPF 50% EC + cypermethrin 5% EC, CPF 35% EC + fipronil 3.5% EC and CPF 30% EC + bifenthrin 3% EC [2].

Although, CPF is moderately toxic to humans and inhibition of plasma cholinesterase is dose-dependent [3,4], number of fatalities owing to deliberate ingestion of pesticide formulations containing CPF are increasingly reported in our laboratory. This may be due to wide application of this pesticide in agriculture and ease of availability to both farming and non-farming community. The most common route of exposure in most

of the reported suicidal deaths is ingestion of commercial formulations containing CPF. Following high dose oral exposure to CPF, as normally expected in suicidal/accidental exposures, rapid absorption and distribution to the brain was observed [5]. CPF is mainly metabolized through oxidative desulfuration to CPF-oxon (O, O-diethyl-O [3,5,6, trichloro-2-pyridinyl] phosphate), the principal toxic metabolite which is primarily responsible for inhibition of cholinesterases [4-8]. However, the oxygen analogue is a highly electrophilic intermediate and hydrolyzed rapidly to nontoxic enolic metabolite 3,5,6, -trichloro-2-pyridinol (TCP) by A-esterases such as paraoxonase in the liver and plasma [4-8]. By incubating various amounts of CPF and CPF-oxon in rat and human blood, it was determined that the concentration of CPF remained constant up to 180 min while the oxon rapidly hydrolyzed ($t_{½}$ rat blood ≈ 10 s, $t_{½}$ human blood ≈ 55s) [6]. After oral exposure, only CPF and CPF-oxon are detectable in blood, but initial clearance of CPF from blood is very rapid [5]. Due to extensive binding of CPF to plasma proteins and distribution in poorly perfused adipose tissue, some fraction of CPF may have slower elimination rate [8,9]. In both occupational and non-occupational exposure to CPF, TCP is a nonspecific biomarker because TCP also is a metabolite of CPF-methyl [8]. Moreover, sensitive analytical methods are required to quantify these metabolites in biological fluids [10].

A few cases of acute fatal poisoning due to the inhalation or ingestion of CPF have been reported by the authors [11-14]. Very few studies have attempted to measure CPF in blood. Literature survey revealed that Gas Chromatography (GC) and High–Performance Liquid Chromatography (HPLC)/Liquid Chromatography (LC) with different detectors are the most widely used methods for the determination of CPF from blood/whole blood [6,7,9-12,15-18]. GC requires extensive sample cleanup procedure for samples with high lipid content to remove fats and oils [19]. The cleanup requirements are much simpler when using GC–Mass Spectrometer (MS), but pre–derivatization steps are still needed [20,21]. Sample manipulations needed in all methods based on GC–MS make the analytical process less robust and time-consuming [20]. GC–MS analysis of acetonitrile extracts seem to be more troublesome due to the degradation of the GC column phase by the polar solvent and the poor focusing of chromatographic peaks due to high acetonitrile polarity [22]. HPLC methods are time-consuming and involve large quantities of solvents whose decantation is another issue of biosafety in the open environment. Liquid chromatography with tandem mass spectrometry (LC–MS/MS) represents a suitable methodology to overcome these limitations. Nevertheless, eliminating matrix interferences in LC–MS is important for accurate quantitative analysis [23]. LC–MS methods require costly instrumentation and qualified technicians and are not widely available in any laboratory [24]. In many problems LC and Thin–Layer Chromatography (TLC) is based on the same concept of separation; they differ only in how their mobile phase is moved (pressure/capillary action) and in how their stationary phase is configured (column/thin layer). While the chromatographic component is rigidly attached to the detector in LC, TLC is carried out in a chamber that is independent of the detector. For this purpose, TLC is more versatile for rapidly evolving problems of chromatographic separation and the development of new separations, usually a TLC–ultraviolet (UV) densitometer is not reliant on the separation systems used [25].

In most of the pesticides related postmortem toxicological cases, especially in deliberate self poisoning with immediate deaths, a high concentration of the causative agent can normally be expected in blood, even though there is rapid metabolism of most of the organophosphorus pesticides (OPPs) in the initial stage [26]. Moreover, in many judicial courts, the identification and confirmation of parent pesticide must be proven rather than their metabolites [27]. In most of the postmortem toxicological situations whole blood will be forwarded to forensic science laboratories to rule out or to confirm the presence of possible intoxicant, because from such samples serum/plasma cannot be obtained. Analysis of whole blood provides many advantages over urine or other biological fluids as elaborated by many of the authors [9,10,15,16,26,28].

Literature survey revealed very few CPF poisoning cases published in which analytical findings are included and further the use of High–Performance Thin–Layer Chromatography (HPTLC) for the assay of CPF in blood is very scant. Modern thin-layer chromatography HPTLC is an instrumental technique that is comparable by its accuracy and precision with both GC and HPLC. Additionally, HPTLC has several preferences over



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HPLC and other techniques [29-33]. Some of the advantages include: concurrent sample and standard processing leading to better analytical precision and accuracy and less internal standard requirement; low sensitivity to impurities; no interference from previous analysis is possible since fresh stationary phases are used for each analysis; the convenience of specific derivatization and multiple detection possibilities without repeating the chromatography; mobile phase consumption per sample is extremely low; instrumentation is simple, relatively inexpensive and easy to handle; many samples on the same plate can be separated in parallel, resulting in a high throughput and rapid low cost analysis; most of the time, little or no cleanup is necessary; the cost of quantitative analysis is only 35% of the cost of HPLC analysis; and no prior treatment for solvents like filtration and degassing. In this paper, a HPTLC method for the determination CPF in human whole blood was developed, validated, and applied to evaluate the fatal levels of CPF in seven fatal cases of poisoning.

## EXPERIMENTAL

### Standard solutions, materials and chemicals

CPF was obtained from Sigma-Aldrich (St. Louis, MO, USA). CPF was dissolved in methanol to obtain standard stock solution of 1mg/mL and stored at 4°C until use. Working solutions were prepared by appropriate dilutions of the stock solution for obtaining neat chromatograms. Aluminum foil TLC plates coated with silica gel 60 $F_{254}$ (10 cm × 20 cm) were obtained from Merck (Darmstadt, Germany). All the chemicals and solvents used in this study were of analytical-grade purity (Merck). Human whole blood was acquired from autopsied corpses at the Raichur Institute of Medical Sciences, Karnataka (India). It was stored in a freezer at -20°C for further use. Blood was analyzed prior to use to ensure no interferences were present.

### High-performance thin-layer chromatography

The instrument consisted of a CAMAG HPTLC system (Muttenz, Switzerland) equipped with Linomat 5 sample applicator with 100µL syringe and TLC Scanner 3. Instrument control, data acquisition and processing were performed using win CATS 1.4.2 software. The samples were applied on TLC plates as 6 mm bands, 15 mm apart, 22 mm from the edges, and 10 mm from the bottoms of the plates. The sample application volume was 10µL/spot. The mobile phase consisted of *n*-hexane-acetone (9+1, *v*/*v*) was used as described in our earlier paper [33]. Plates were developed to 8 cm from the lower edge of the plate. After development, mobile phase components were evaporated using air-dryer. *In situ* densitometric scan of the separated bands was performed at 295 nm under the following settings: absorption mode in the UV region; slit dimension of 4.00 mm × 0.45 mm; scanning speed of 20 mm/s, data resolution 100µm/step; and deuterium ($D_2$) light source was used. Spectrum scan was done in absorption mode in the UV region 200-400 nm using $D_2$ lamp, slit dimension of 4.00 × 0.45 mm, spectrum scan speed of 20nm/s and data resolution was set to 1 nm/step. Peak areas were recorded for all the separated bands.

### Sample preparation

Postmortem blood, blood calibrators, and control blood samples (one milliliter each) were processed in a 15mL screw–capped polypropylene tube as follows. To each specimen, 5mL of acetonitrile was added and vortex-mixed for 2 min. 1mL of phthalate buffer solution pH 5 and 5mL of extraction solvent toluene were added to this mixture, and the solutions were again vortex-mixed for 2 min. After centrifugation at 4000 rpm for 10 min, the organic layer was evaporated to dryness under vacuum using sample concentrator and the resulted residue was reconstituted with 0.5mL of methanol.

### Validation parameters

The method is validated as per the FDA guidelines for bioanalytical method validation in terms of selectivity, linearity (calibration curve), sensitivity (lower limit of quantification), accuracy, precision (within-day and between-day repeatability), recovery, reproducibility, carry-over and stability [34]. These parameters are discussed in results and discussion.

## RESULTS AND DISCUSSION

### Liquid–liquid extraction

Liquid–Liquid Extraction (LLE) is among the most used and useful methods in the preparation of biological samples. This is due to a variety of features like simplicity, rapid method development, and reasonable selectivity. One point to consider is that unlike solid–phase systems, LLE systems are more likely to give consistent results year after years, since there is typically less variability in batch to batch with solvents [35,36].

Rosario García–Repetto reported that the most used methods for extraction of pesticides from human samples still are LLE, solid–phase extraction and solid–phase microextraction. The published papers in the last 10 years revealed several examples of LLE procedures applied in cases of lethal poisoning by pesticides in forensic science laboratories [37]. Several methods for extracting pesticides from body fluids which generally involve the use of LLE with water-immiscible organic solvents and wide ranges of pH starting from pH 3.0 to pH 7 were employed and variable extraction yields were reported. This convinced us to develop an extraction method.

Extraction parameters such as extraction solvent and sample pH were optimized using 1mL of spiked whole blood samples at a concentration of 10µg and 50µg of CPF. Six organic solvents dichloromethane (DCM), diethyl ether, ethyl acetate, hexane, *tert*-butyl methyl ether (TBME) and toluene were assessed based on preliminary studies to obtain maximum extraction yield of CPF. In addition, extraction procedure involving protein precipitation with 5mL of acetonitrile (ACN), followed by centrifugation, aspiration and evaporation, was also tested. OPPs are generally more stable in acidic pH ranging from 3 to 6 and are decomposed in alkaline media [38]. The change in the pH value of aqueous phase will change the ionization form of certain analytes and there it will affect their water solubility and extractability. Therefore, the effect of sample pH on the extraction yield of CPF was assessed in the pH range 3 to 7. In each solvent and at each pH, experiments were performed in triplicate.

The results indicated that the LLE procedure using toluene as extraction solvent yielded better extraction yield with an average recovery of 87.8% and the Relative Standard Deviation (RSD) was lower than 1.27% at both the concentrations. The use of toluene as the extraction solvent provided excellent extraction efficiency and sharp chromatography peak. Protein precipitation by ACN resulted in drastic decrease in endogenous substances, and the efficiency of extraction was further improved by the addition of toluene as extraction solvent. Extraction efficiency decreased in the order of ethyl acetate, ACN, TBME, DCM, diethyl ether, and hexane. Recovery of CPF in different solvents ranged from 40.88% to 87.80%. The obtained recovery in each solvent is presented in Table 1. Hence, toluene extraction procedure was adopted for further studies.

Table 1: Extraction recovery of chlorpyrifos in different solvents.

| Extraction solvent | 10 µg/mL (n = 3) | | 50 µg/mL (n = 3) | | Average Recovery % |
|---|---|---|---|---|---|
| | Recovery [%] | RSD [%] | Recovery [%] | RSD [%] | |
| Acetonitrile | 78.68 | 0.94 | 83.68 | 1.35 | 81.18 |
| Dichloromethane | 62.10 | 1.19 | 62.64 | 1.80 | 62.37 |
| Diethyl ether | 59.32 | 1.25 | 60.67 | 1.86 | 60.00 |
| Ethyl acetate | 80.60 | 0.92 | 84.07 | 1.34 | 82.34 |
| Hexane | 39.62 | 1.87 | 42.13 | 2.68 | 40.88 |
| *tert*-butyl methyl ether | 78.82 | 0.94 | 80.43 | 0.86 | 79.63 |
| Toluene | 86.71 | 0.85 | 88.88 | 1.27 | 87.80 |

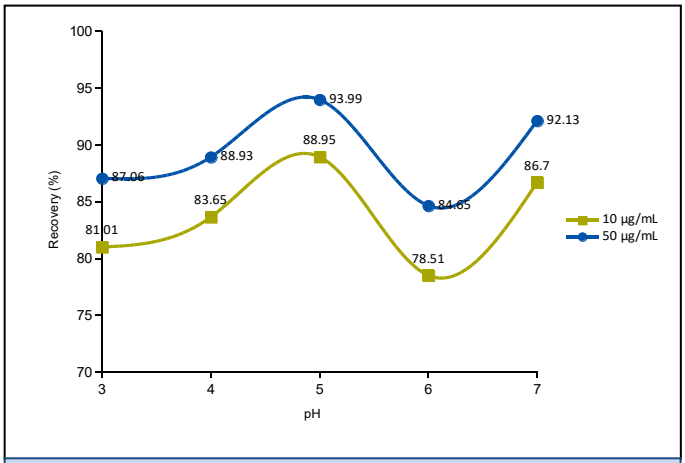


Figure 1: Extraction yield obtained for chlorpyrifos at different pH using toluene as extraction solvent at 10 and 50µg/mL.

The effect of sample pH on the extraction yield of CPF was studied at different pH ranging from pH 3 to 7 using the toluene extraction procedure. From the comparison presented in Figure 1, it is evident that recovery of CPF from whole blood is maximum at pH 5 with an average analytical recovery of 91.47% and the RSD was less than 1.20% at both the concentrations. Extraction efficiency decreased in the order of pH 5, pH 7, pH 4, pH 3 and pH 6. Recovery of CPF at different pH ranged from 81.58% to 91.47%. The significant increase in extraction yield obtained at pH 5 may be attributed to enhanced suppression of ionization of CPF at this

pH. Hui *et al* investigated the hydrolysis of CPF in buffered aqueous media at different temperature and pH conditions. Their study on rate constants and half-life revealed that CPF was relatively stable in acidic medium, and the rate of degradation increased as the pH increased [39]. Similar observations were also reported by another study in which half-life of CPF was maximum at pH 4.7 (63 days) followed by pH 6.9 (35 days) and pH 8.1 (23 days) indicating the increased hydrolysis of CPF in alkaline medium [40]. Majority of the blood samples received in our laboratory have pH in the range of 5 to 7, which suggest that the blood pH favored the chemical stability of CPF as indicated by the obtained recovery yield of 91.47% at pH 5.

**Validation study**

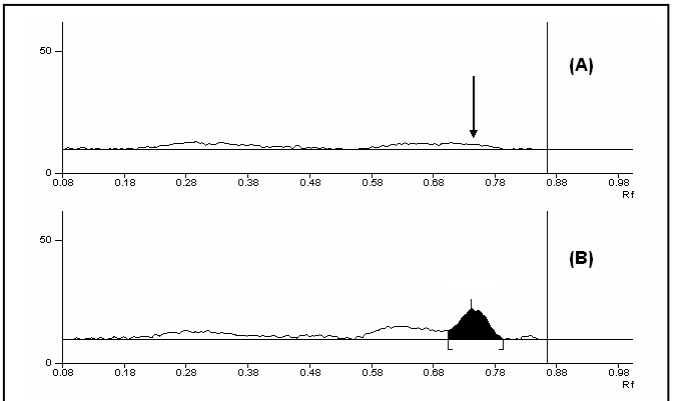


Figure 2: (A) HPTLC-UV densitogram obtained from blank blood; (B) blank blood spiked with LLOQ of chlorpyrifos.

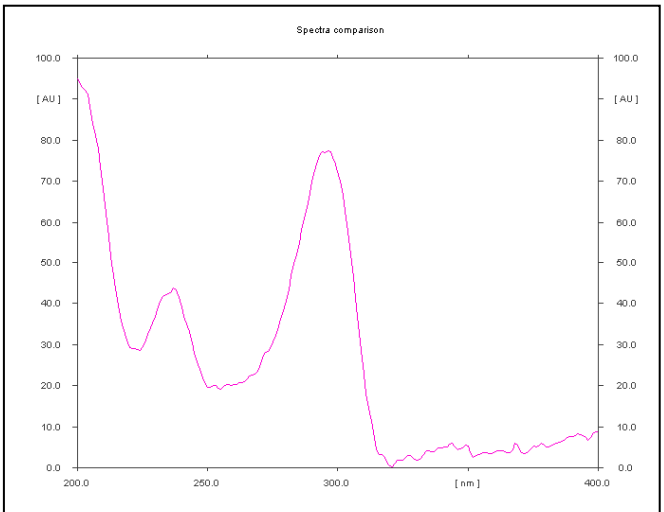


Figure 3: UV spectrum of chlorpyrifos at LLOQ (2µg/mL).

Selectivity of the method was investigated by evaluating six different blank whole blood samples and comparing the chromatograms acquired with those acquired by spiking the samples at low analyte concentration (2µg/mL). The chromatograms obtained for blood samples using *n*-hexane–acetone (9:1, v/v) as mobile phase were simple, showing CPF (retardation factor, $R_f$ = 0.74 ±0.01) as the main components with good resolution between pesticide peak and the nearest adjacent peak (peak resolution, $R_S \geq 1.25$). For such samples, a UV spectrum was obtained to ensure peak purity. The peak purity of CPF from blood samples conformed fully with that of the corresponding standards. It was thus established that no impurities or degradation products co-eluted with the CPF peak. No interferences by matrix constituents were observed at the $R_f$ value of analyte (Figure 2). Spectrum scan of the CPF band in absorption mode in the UV region 200-400 nm indicated that the UV apex of maximum absorption was 295 nm. The obtained UV spectrum of CPF at 2µg/mL is presented in Figure 3. Thus, the measurements at $R_f$ value 0.74 and detection wavelength 295nm offered selective detection of CPF free from interference.

The linearity of the method was established by analysis of blank blood spiked with CPF in the concentration range of 2 to 100µg/mL and each calibration level was analyzed in five replicates. The average recoveries of CPF blood calibrators ranged from 86.61% to 94.21% with an average analytical recovery of 90.1%. Accuracy expressed as bias [%] (bias [%] = (measured concentration-nominal concentration)/nominal concentration × 100) ranged from -5.79% to -13.5% and RSD was less than 2.57% at all the six calibration points. The RSD values were within the limit of acceptable variability as per the FDA guidelines for bioanalytical method validation. The recovery results of blood calibrators of CPF did not deviate more than 15% of nominal concentration including the concentration at lower calibration point. The linear regression analysis of the CPF peak area in spiked blood against CPF concentration resulted in a linear calibration curve in the range of 2-100µg/mL ($y = 117.9x + 411.66$ with $r^2 = 0.9989$). The obtained parameters of blood calibration curve for CPF were satisfactory with correlation coefficients ($r^2$) = 0.9989. The good linearity of the calibration plot and negligible scatter of experimental points are evident from the values of correlation coefficient and standard deviation of the residues (6.6%).

Sensitivity (lower limit of quantification, LLOQ) was determined by analyzing seven replicates of spiked samples at 2µg/mL.

The calculated LLOQ for CPF in blood was 1.97µg/mL (RSD = 0.6% and bias = -12.5%). The LLOQ depend upon nature of the matrix and method of analysis. The reported LLOQ in our method was considered satisfactory, taking into consideration the high pesticide concentrations expected in acute fatal intoxication cases [12,26]. As per the FDA guidelines, the analyte peak (response) should be identifiable, discrete, and reproducible, and the back-calculated concentration should have precision that does not exceed 20% of the CV and accuracy within 20% of the nominal concentration. The obtained results demonstrated that the precision (RSD [%]) and accuracy (bias [%]) values are within the limit of acceptable variability.

Accuracy was estimated by means of recovery experiments. Percentage accuracy was determined (using results from assessment of the precision) as the closeness of results for spiked samples to the nominal value of in-house standards. Percentage accuracy was reported as bias [%]. The precision of the method was determined as the repeatability of the recoveries at each fortification level, within and between days. Precision was reported as relative standard deviation (RSD = (standard deviation/mean) × 100). The within–day precision and between–day precision was carried out at five independent extractions of CPF at 2, 10 and 50µg/mL in one day and on three different days, respectively. The within–day precision in blood samples ranged from 0.80 to 2.57%, and accuracy ranged from 6.94 to 13.5%. The between–day precision ranged from 0.68 to 2.14%, and accuracy ranged from 6.9 to 14%. The within–day and between–day accuracy (% bias) was within 14% and the within–and between–day precision (RSD) was less than 2.57% at the three concentrations. Hence, the obtained results demonstrate that values were within the limit of acceptable variability for bioanalytical method validation and the method is accurate and precise. The average recovery of CPF obtained by analyzing samples within–and between–day intervals was found to be 89.39%. Validation data for precision and recovery are presented in Table 2. Percentage recovery depends on the matrix, extraction solvents, method of analysis, and the amount to be analyzed [41]. Recovery of CPF obtained in our method was acceptable, which lies within the reported values in the literature, taking into consideration the analysis of CPF from more complex matrix, the whole blood.

Table 2: Accuracy and precision data.

| Theoretical concentration µg/mL | Experimental concentration µg/mL | Recovery [%] (mean ± sd) | RSD [%] | Bias [%] |
|---|---|---|---|---|
| Within-day (n = 5) | | | | |
| 2 | 1.73 | 86.61 ± 2.23 | 2.57 | -13.5 |
| 10 | 8.87 | 88.71 ± 0.71 | 0.80 | -11.3 |
| 50 | 46.53 | 93.06 ± 1.81 | 1.94 | -6.94 |
| Between-day (n = 15) | | | | |
| 2 | 1.72 | 86.27 ± 1.85 | 2.14 | -14.0 |
| 10 | 8.85 | 88.55 ± 0.60 | 0.68 | -11.5 |
| 50 | 46.55 | 93.11 ± 1.17 | 1.26 | -6.9 |
| Average | | 89.39 | | |

Reproducibility of sample application on the TLC plates and UV-densitometric scan of the separated CPF band was studied at 50µg/mL. Each sample was applied on the TLC plates five times using automated sample applicator equipped with a 100µL syringe. After chromatography, the separated pesticide band was repeatedly scanned for five times and the obtained peak areas were recorded. Reproducibility of sample application and densitometric scan of CPF band was 2.21% and 0.21%, respectively. Sample carry–over was performed by using the syringe rinsed with methanol by injecting a blank blood sample after the injection of Upper Limit of Quantification (ULOQ) (100µg/mL) of the calibration curve and studying the obtained chromatogram of blank blood sample. The obtained chromatogram of blank blood sample indicated that the sample carry–over effect was not observed for CPF after the injection of ULOQ of the calibration curve.

Stability of CPF in spiked blood at two concentration 25 and 100µg/mL indicated that there was no significant decrease in the concentration of CPF in the stability samples under different storage conditions such as freeze and thaw stability for three cycles, bench-top stability at room temperature for 4 h, long-

term stability at 4°C for 1 month, and processed sample stability at room temperature for 6h. Percentage stability was expressed as accuracy (%) which is calculated as (mean response of stability sample/mean response of comparison sample) × 100. Overall, the measured concentrations of CPF under different storage conditions did not deviate more than 4.76% from the nominal concentration. Many OPPs are unstable in blood, because of their degradation by esterase activity [42,43]. But on the other hand, organophosphorothioates like CPF are stable in blood and are not metabolized by these enzymes since proteins and lipids present in the biological specimens may stabilize these compounds [43]. The results of the stability study were summarized in Table 3. The standard CPF solution was found to be stable at room temperature and at 4°C with RSD less than 3.78%. CPF was found to be stable on the TLC plates (silica gel 60 $F_{254}$) at 0h and after 3h and 6h with RSD less than 1.61%.

Table 3: Results of stability study.

| Stability | 25 µg/mL (n = 3) | | 100 µg/mL (n = 3) | |
|---|---|---|---|---|
| | Accuracy [%] | RSD [%] | Accuracy [%] | RSD [%] |
| Freeze and thaw stability (3 cycles) | 102.52 | 4.76 | 103.30 | 3.03 |
| Bench-top stability (4 h) | 100.12 | 0.20 | 100.07 | 0.03 |
| Long-term stability (1 month) | 105.20 | 3.42 | 101.45 | 2.15 |
| Processed sample stability (6 h) | 101.6 | 0.55 | 102.00 | 0.28 |

**Application of the method to fatal intoxication cases**

*Case 1*: A 55-year-old female was found dead in her house with a suspected homicidal poisoning by insecticide. The autopsy was conducted 8h after the occurrence of death. An unlabeled pesticide container of 1L capacity with about 1mL of pale-yellow color liquid having insecticide smell was also forwarded for chemical examination.

*Case 2*: A 34-year-old man was found dead in his house with a history of suspected homicidal poisoning. Autopsy was performed 8h after noticing the dead body.

*Case 3*: A 25-year-old female attempted suicide by consuming an unknown pesticide due to severe abdominal pain and died after 2h of treatment. The autopsy was conducted 6h after the occurrence of death.

*Case 4*: Under the influence of alcohol, a 50-year-old man ingested an unknown amount of pesticide solution and died after 7h of treatment. The autopsy was carried out 15h after the occurrence of death.

*Case 5*: A 45-year-old male farmer ingested an unknown amount of pesticide solution of Durmet® containing 50% of CPF, in a suicide attempt due to agriculture losses. He was declared dead after 4h of treatment and autopsy was conducted 10h after the occurrence of death.

*Case 6*: In a suspected case of assault and murder, a 73-year-old male was admitted to the hospital and died 6 h after treatment. The autopsy was carried out 9h after the occurrence of death. Stomach contained about 150mL of whitish liquid with kerosene like odor. A plastic cover containing about 240g of ash color powder with some black color crystals was recovered by the investigation officer from the crime scene and forwarded to our laboratory for chemical examination.

*Case 7*: A 71-year-old male was found dead in the railway platform with an unknown history. Autopsy was performed 8h after noticing the dead body.

In case 1, case 3, case 4, case 5 and case 7, postmortem blood along with other autopsy samples such as stomach and its contents, portion of liver, and portion of kidney were sent for toxicological analysis. In case 2 and case 6, excluding postmortem blood all other autopsy samples were sent. A systematic toxicological screening for pesticides in the autopsy samples was conducted using TLC, HPTLC–UV densitometry and GC–MS (GC 2010 gas chromatograph equipped with an AOC 20i autosampler and coupled to QP 2010 mass spectrometer, Shimadzu, Japan) methods following LLE. TLC followed by post chromatographic derivatization with palladium chloride reagent and bromophenol blue reagent indicated the presence of pesticides of organothiophosphate group in all the seven mentioned medico–legal cases. Presence of CPF was confirmed by HPTLC–UV scan with the library match and GC–MS Electron Ionization (EI) full scan screening. A high concentration of CPF was detected in the stomach contents, indicating the oral administration of the pesticide solution. The proposed HPTLC



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method was used for the quantification of CPF in postmortem blood samples. The estimated concentrations were 20.07µg/mL in case 1, 18.68µg/mL in case 3, 5.92 µg/mL in case 4, 13.26 µg/mL in case 5, and 31.44 µg/mL in case 7. The representative UV densitogram, UV spectrum and library match obtained for postmortem blood sample of case 4 was shown in Figure 4. In two cases (case 2 and case 6), postmortem blood was not available for quantification of CPF, and the present method only helped to identify the ingested pesticide. In case 6, phorate was detected in the ash color powder recovered from the scene of offense and it was absent in the autopsy samples, in which only CPF was positively identified. Confirmation of the HPTLC results was done by analysis of postmortem blood samples by GC–MS. Quantification of blood alcohol by headspace GC–FID (Agilent, 7697A Headspace sampler with 7890B GC) revealed a Blood Alcohol Concentration (BAC) of 72 mg/100 dL in case 4 and 115 mg/100dL in case 5, respectively.

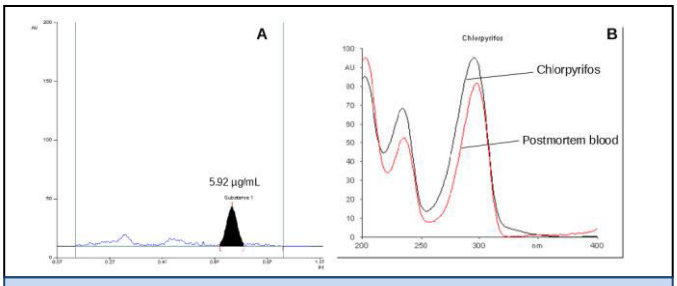


Figure 4: (A) HPTLC-UV densitogram obtained from a postmortem blood sample of case 4; (B) Overlaid UV spectrum of a standard chlorpyrifos and postmortem blood sample of case 4 from a chlorpyrifos fatal poisoning case determined at 5.92µg/mL.

Out of the 7 medico–legal cases, times of both ingestion and death were known in 4 cases and deaths from ingesting CPF were within 7h of ingestion (average 4.75h) as a result of acute oral exposure. Similar observations were reported by Eddleston *et al* [44]. Despite rapid metabolism of CPF in humans, the reported high concentrations of CPF in case 1, case 3, case 5, and case 7 may be due to the ingestion of large amounts of pesticide solutions by the deceased persons, which is normally observed in suicidal attempts [45]. The study conducted by Meuling *et al* has shown that the urinary excretion of TCP was not complete within 120h after dosing, indicating the accumulation of CPF or TCP in the body [3]. In addition, CPF is lipophilic and the portion of the compound that partitions in body fat can be eliminated more slowly, although the blood clearance rate is rapid in the initial stage [9]. The low concentration of CPF determined in case 4 may be attributed to the treatment underwent by the deceased before death.

A range of 10–100µg/mL in human tissues reflects the acute accidental or intentional exposure to OPPs [46]. Several authors have reported different concentrations of OPPs in fatal cases of poisoning in humans. The concentrations of OPPs detected in each case depends upon number of factors such as, quantities of pesticide consumed; time gap between ingestion and death; intervals between time of death, autopsy and laboratory analysis; site from which blood sample obtained during autopsy; storage conditions; and stability of the analyte in the sample [47]. The reported concentrations of CPF in fatal cases ranged from 0.21 to >25µg/mL [11,12,14]. In our study, the concentrations of CPF in postmortem blood samples of case 1, case 3, case 5 and case 7 are within the range of fatal levels, which helped to conclude that the death was due to an acute intoxication with a large overdose of pesticide. Compared to serum/plasma, the whole blood is the most appropriate matrix for estimating the total concentration of causative pesticide because of plausible strong binding of pesticides at erythrocytes [16]. Determination of OPPs level in biological specimens such as blood sample is a challenging task because these pesticides have been stated to remain in the blood circulation for a short period [9,48].

## CONCLUSION

The optimized extraction procedure followed by a validated HPTLC method is simple, rapid and accurate which is particularly helpful in forensic toxicological analysis of CPF in postmortem blood and in emergencies for making treatment decisions. The reported concentrations of CPF in postmortem blood offers conclusive scientific evidence for diagnosis of death and help the toxicologists in the interpretation of fatal poisoning cases with OPPs.

## ACKNOWLEDGMENTS

The authors are thankful to the Director, Directorate of Forensic Science Laboratories, Bengaluru, Karnataka, India and Deputy Director, Regional Forensic Science Laboratory, Kalaburagi, Karnataka, India, for their support.

## AUTHOR'S CONTRIBUTION

All the authors designed and coordinated the study. Dr. Praveen U Sanganalmath and Dr. Purigali M Nagaraju performed the experiments and analyzed the data. Dr. Kuruba Sreeramulu and Dr. Praveen U Sanganalmath drafted the manuscript. All authors contributed to the final text and approved it.

## DECLARATIONS

Funding: No funding was received for this work.

Conflicts of interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with living human participants or animals.

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