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ORIGINAL RESEARCH PAPER

Development and validation of a simple and rapid thin-layer chromatography–UV densitometry method for the determination of triazophos in human whole blood for forensic toxicological applications

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Abstract
No published studies reported triazophos (TZP) analysis in human whole blood following fatal intoxication. In this study, a method for the determination of TZP in human whole blood using liquid–liquid extraction (LLE) and thin-layer chromatography (TLC)–ultraviolet (UV) densitometry is described. Chromatography was performed on silica gel $60F_{254}$ TLC plates using mobile phase *n*-hexane:acetone (8:2, *v/v*) and UV densitometric detection at 248 nm. Better extractions were achieved by using ethyl acetate solvent at pH 5 with a recovery of 92.17%. Calibration curve for TZP in blood was linear from 2 to 100 μg/mL (0.04 to 2 μg/spot) and the sensitivity of the method (LLOQ) was 2 μg/mL (0.04 μg/spot). The method showed excellent within-day precision (0.37 to 0.82%, RSD) and between-day precision (0.39 to 1.47%, RSD) for spiked blood samples at 2, 10, and 50 μg/mL. The measured concentrations of TZP in blood did not deviate more than 2.69% under different storage conditions. TZP concentrations in two fatal cases of poisoning were reported. To the best of our knowledge, this is the first validated TLC–UV densitometric method developed to determine TZP in human whole blood.

Keywords Thin-layer chromatography · Liquid–liquid extraction · Postmortem blood · Triazophos

Abbreviations

EC	Emulsifiable concentrate
TLC	Thin-layer chromatography
LLE	Liquid–liquid extraction
OPPs	Organophosphorus pesticides
RSD	Relative standard deviation
TZP	Triazophos

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1 Introduction

One of the most difficult tasks in the forensic toxicological analysis is to identify unknown or unsuspected poisons in fatal cases of poisoning. A general extraction and screening method has been used to identify various organophosphorus pesticides (OPPs) in forensic samples, but these techniques have not been established or optimized to selectively extract and identify the precise causal OP compound. OPPs, because of their ease of availability and potential toxicity, are commonly used as sources of poisons in homicides, suicides, and also in cases of deaths due to accidental poisoning [1–4]. Poisoning incidents due to OPPs could not be determined on the basis of clinical symptoms alone, and, in most cases, the presence of an OPP must be demonstrated in the blood and organs before it can be deemed a cause of death. In different intoxication cases by OPPs, the pathological data are not specific and identical and in such situation the determination of causal pesticide in blood would render great help in the diagnosis

of death [5]. The assay of plasma and red cell cholinesterase activity is one of the most widely used devices for determining human exposure to OPPs. But in forensic cases, the acidic nature of postmortem blood poses difficulty in the determination of esterase activity [4, 6]. Moreover, the addition of sodium fluoride as a preservative for most of the biological fluids in the forensic toxicological analysis is known to be a deactivator of cholinesterase [4, 7]. Furthermore, indication of OPP exposure is usually drawn from the measurement of dialkyl phosphate metabolites in urine. But the identification of the causal OPP agent cannot be ascertained by this approach, since many OPPs are converted to these non-specific metabolites [1, 8, 9].

Triazophos (O,O-diethyl-O-1-phenyl-1H-1,2,4-triazol-3-yl-phosphorothioate, TZP) is one of the widely used broad-spectrum OPPs in India. TZP is the active ingredient in many commercial pesticide formulations available in India registered for pest control in various crops such as cotton, rice, and soybean [10–12]. Products containing the active substance TZP are commercially available as single active emulsifiable concentrate (EC) formulations (20% EC and 40% EC) or containing a combination of active ingredients of TZP and deltamethrin (35% EC + 1% EC).

Several fatal and nonfatal cases of human poisoning have been reported in our laboratory owing to the ingestion or inhalation of TZP. Literature study disclosed a lack of published cases of TZP poisoning that include analytical results. In addition, methods that focus on determining different OPPs in human blood are still scarce, and the fatal levels of different OPPs in humans remain largely unknown.

A few numbers of analytical methods have been described for the determination of residues of TZP in stomach content by thin-layer chromatography (TLC)–mass spectrometry (MS) [13]; urine, blood, serum, and food samples by gas chromatography (GC)–nitrogen phosphorus detector (NPD) and GC–MS [14]; duck meat by fluorescence spectroscopy [15]; liver from wildlife specimens by GC–MS/MS [16]; plasma by GC–MS [17]; blood serum by GC–MS/MS [18]; and in human blood by liquid chromatography (LC)–MS/MS [19]. No published studies have reported the analysis of TZP in human whole blood following fatal intoxication. Whole blood must be analyzed in postmortem toxicology, as plasma or serum cannot be derived from such samples. The advantages of using blood as a choice of matrix for the determination of parent compound were well explained by various authors [1, 5, 6, 8, 20, 21]. Therefore, it may be of interest to develop procedures for the determination of TZP in human whole blood samples. In this study, we present a simple and reliable method for the determination of TZP in human whole blood using TLC coupled with ultraviolet (UV) densitometry. To our knowledge, this is the first validated TLC–UV densitometric method developed to determine TZP in human whole blood samples, which is especially useful in a forensic toxicological analysis. Liquid–liquid extraction (LLE) procedure was optimized for extraction solvent and sample pH for achieving better extraction efficiency of TZP from spiked blood.

2 Experimental

2.1 Chemicals, reagents, and standard solutions

TZP was obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetone, acetonitrile, dichloromethane, diethyl ether, ethyl acetate, hexane, methanol, *tert*-butyl methyl ether, and toluene were of analytical grade (Merck, Darmstadt, Germany) purity. 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. Before use, blood was analyzed to ensure that no interferences were present.

The stock solution of TZP (1 mg/mL) was prepared in methanol. The working standard solution (0.1 mg/mL) was prepared by diluting the stock solution in methanol. Stock solution and working standard solutions were stored in a refrigerator at a temperature below 4 °C until use.

2.2 Sample preparation

One milliliter of each specimen postmortem blood, blood calibrators, and control samples was processed in a 15-mL screw-capped polypropylene tube as follows. To each specimen, 5 mL of acetonitrile was added and vortex-mixed for 2 min. To this mixture, 1 mL of phthalate buffer solution pH 5 and 5 mL of extraction solvent ethyl acetate were added and again vortex-mixed for 2 min. The solutions were centrifuged for 10 min at 4000 rpm to obtain clear supernatant. The organic layer was aspirated and evaporated to dryness under vacuum using a sample concentrator. The resulted residue was dissolved in 0.5 mL methanol.

2.3 Instrumentation

The instrument used was a CAMAG HPTLC system (Muttenz, Switzerland) consisting of Linomat 5 sample applicator, glass twin-trough chamber, Reprostar 3 digital documentation system, and TLC Scanner 3. Instrument control and data acquisition and processing were performed using winCATS 1.4.2 software (CAMAG).

The samples were applied on TLC plates under the following settings: 100-μL syringe with N_2 flow, application volume of 10 μL, dosing speed of 100 nL/s, sample bandwidth of 6 mm, distance between the tracks of 15 mm, distance from

the lower edge of the plate of 10 mm, and distance from the side of a plate of 22 mm. The mobile phase consisted of *n*-hexane:acetone (8:2, *v/v*) was used. Plates were developed to a distance of 8 cm from the lower edge of the plate. After development, mobile phase components were evaporated using air-dryer and the developed plates were photo-documented by the illumination of the plates at 254 nm and 366 nm. In situ densitometric scan of the separated bands was performed at 248 nm under the following settings: absorption mode in the UV region, slit dimension of 4.00 × 0.45 mm, scanning speed of 20 mm/s, data resolution of 100 μm/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 20 nm/s, and data resolution of 1 nm/step. Peak areas were recorded for all the separated bands.

2.4 Validation parameters

Validation for selectivity, linearity, sensitivity, accuracy, precision (within-day and between-day repeatability), recovery, reproducibility, carry-over, and stability was performed in compliance with international standards using appropriate statistics [22]. The discussion of these parameters is presented in Section 3.

3 Results and discussion

In order to select the optimum mobile phase, extraction solvent, and sample pH, preliminary experiments were conducted. The mobile phase consisted of *n*-hexane:acetone in the ratio 8:2 (*v/v*) was used as described in our earlier paper [23]. LLE is often used in sample preparation of blood samples as it can provide extracts with low levels of co-extracted matrix material [24]. *Rosario García-Repetto* reported that the mostly used methods for the 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 [25]. LLE provides important benefits in toxicological analysis such as pre-concentration, simplicity, low cost, high speed, simple device, easy operation, and analytical system compatibility [26, 27]. In LLE, selection of suitable extraction solvent and sample pH is the most important parameter to be optimized for obtaining a better extraction yield of the target analyte. Based on the preliminary studies, six organic solvents dichloromethane, diethyl ether, ethyl acetate, hexane, *tert*-butyl methyl ether, and toluene were evaluated to obtain maximum extraction yield of TZP. In addition, the extraction procedure involving protein precipitation with 5 mL of acetonitrile, 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 [28]. Therefore, the effect of sample pH on the extraction yield of TZP was assessed in the pH range 3 to 7.

The extraction procedure involving each solvent was examined at room temperature using 1 mL of spiked whole blood samples at a concentration of 10 μg and 50 μg of TZP. In each solvent, experiments were performed in triplicate. The obtained results indicated that ethyl acetate has provided better extraction efficiency than the rest of the extraction solvents. The average recovery of TZP obtained by ethyl acetate extraction procedure was found to be 89.34%. This

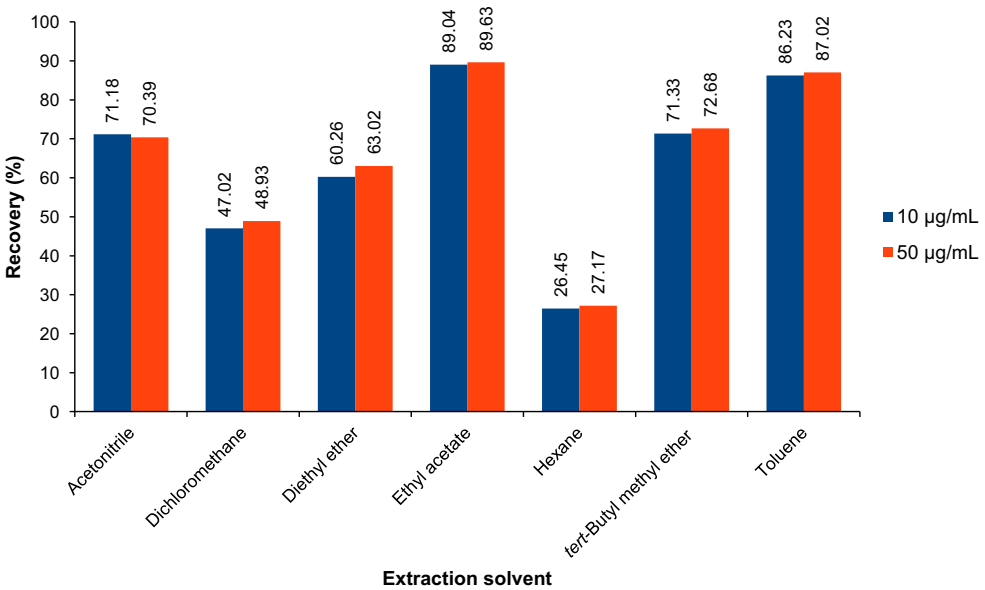

Fig. 1 Effects of different extraction solvents on extraction efficiency of triazophos at 10 and 50 μg/mL

extraction procedure has provided neat chromatograms and sharp chromatography peaks. Protein precipitation by acetonitrile resulted in a drastic decrease in endogenous substances, and extraction efficiency was further enhanced by the addition of ethyl acetate as an extraction solvent. Extraction efficiency decreased in the order of ethyl acetate, toluene, *tert*-butyl methyl ether, acetonitrile, diethyl ether, dichloromethane, and hexane. An effect of different extraction solvents on the extraction efficiency of TZP is shown in Fig. 1. The effect of sample pH on the recovery of TZP was tested at different pH values (pH 3 to 7) using ethyl acetate as an extraction solvent at 10 and 50 μg/mL, in triplicate. The results indicated that the recovery of TZP from whole blood is maximum at pH 5 followed by pH 7, pH 6, pH 3, and pH 4. An average analytical recovery of 92.17% was achieved using ethyl acetate as an extraction solvent and sample pH 5. The extraction yield obtained at different pH is shown in Fig. 2. The substantial improvement in extraction efficiency at pH 5 can be ascribed to the enhanced suppression of TZP ionization at this pH.

3.1 Method validation

Selectivity 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. The chromatograms obtained for blood samples using *n*-hexane:acetone (8:2, *v/v*) as the mobile phase were simple, showing TZP (retardation factor, $R_F = 0.18 \pm 0.01$) as the main component with a good resolution between TZP and the nearest adjacent peak ($R_S \geq 1.25$). All blank blood samples and spiked samples were free of co-eluting peaks at the R_F of TZP. Chromatograms obtained from blank blood, blood spiked with TZP (2 μg/mL), and postmortem blood of case 1 were shown in Fig. 3. The use of mobile phase *n*-hexane:acetone (8:2, *v/v*) offered an excellent separation of

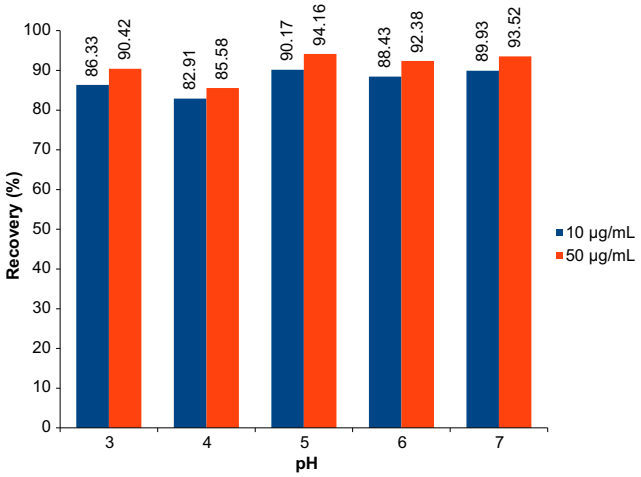

Fig. 2 Extraction yield obtained at different pH values using ethyl acetate as an extraction solvent at 10 and 50 μg/mL

TZP from other OPPs of forensic relevance and other suspected pesticides such as deltamethrin.

The wavelength of maximum absorption was determined to be 248 nm by measurement of the in situ UV absorption spectrum of TZP. To ensure peak purity, a UV spectrum was obtained for the TZP from blood samples. The peak purity of TZP from samples conformed fully with that of the standard. The detection wavelength was selective for TZP and enabled detection free from interference.

The linearity of the method was established by analysis of blank blood spiked with TZP in the concentration range of 2 to 100 μg/mL (0.04 to 2 μg/spot). Spiked blood samples were processed using the described extraction procedure, and each calibration level was analyzed in five replicates. The average values of measured concentrations for TZP were 1.75, 4.41, 9.01, 23.04, 47.04, and 95.45 μg/mL, respectively, at 2, 5, 10, 25, 50, and 100 μg/mL (0.04, 0.1, 0.2, 0.5, 1, 2 μg/spot). The average recoveries of TZP blood calibrators were 87.52, 88.22, 90.09, 92.16, 94.07, and 95.45% with an overall average analytical recovery of TZP being 91.25% and the relative standard deviation (RSD) being less than 0.82% at all the concentrations. The RSD values were within the limit of acceptable variability. The linear regression analysis of the TZP peak area in spiked blood against TZP concentration resulted in a linear calibration curve in the range of 2–100 μg/mL. The regression equation obtained for TZP was $y = 100.9x + 567.1$, and the correlation coefficient (r^2) was 0.9992.

Sensitivity (lower limit of quantification, LLOQ) for TZP in blood, defined as the lowest concentration in the calibration curve with acceptable precision and accuracy (RSD = 0.55% and bias = −12.5%), was 2 μg/mL (0.04 μg/spot) and was determined by analyzing seven replicates of spiked samples. In our method, the reported LLOQ was bound to the analysis of TZP in a complex matrix, the human whole blood.

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 RSD. Percentage accuracy was determined as the closeness of results for spiked samples to the nominal value of in-house standards (using results from the assessment of the precision). Percentage accuracy was reported as error (%). The precision of the method was assessed by analyzing the spiked blood samples within day and between day. The within-day precision and between-day precision were carried out at five independent extractions of TZP at 2, 10, and 50 μg/mL in 1 day and on 3 different days, respectively. The within-day precision in blood samples ranged from 0.37 to 0.82%, and accuracy ranged from 5.92 to 12.5%. The between-day precision ranged from 0.39 to 1.47%, and accuracy ranged from 6.14 to 12%. Precision and accuracy data are presented in Table 1. The average recovery of TZP achieved by analyzing samples within- and between-day intervals was found to be 90.67%.

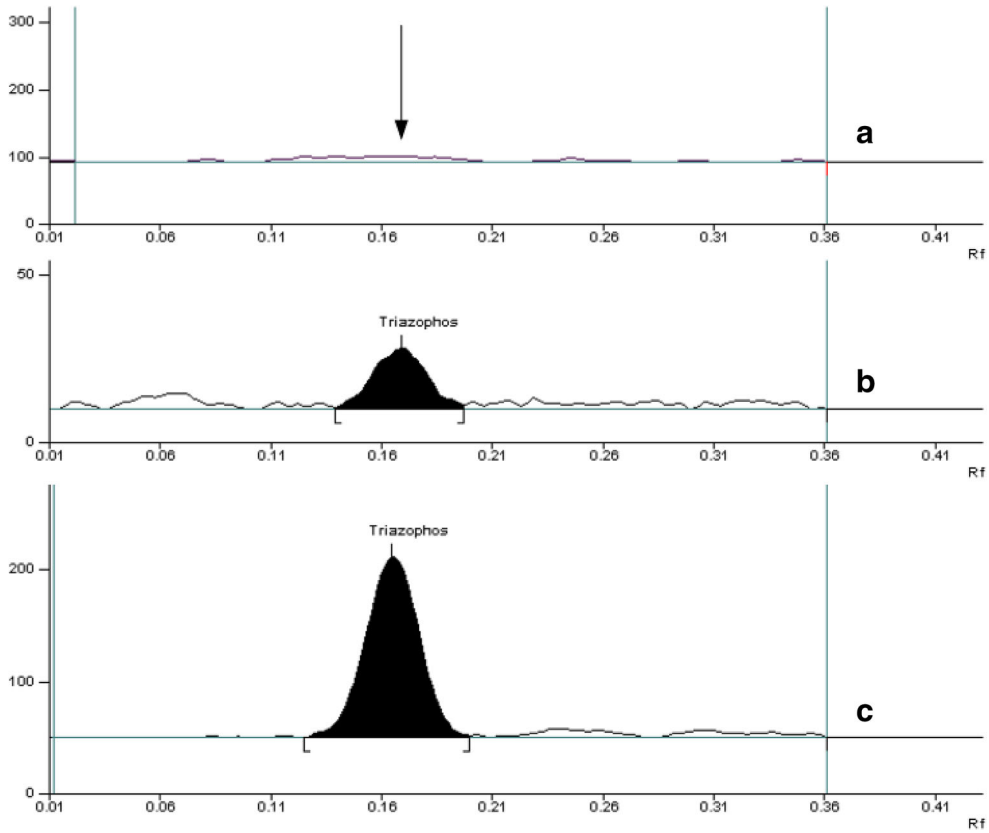

Fig. 3 TLC–UV densitogram obtained from blank blood (**a**), blank blood sample spiked with triazophos (2 μg/mL) (**b**), and postmortem blood of case 1 (**c**)

Reproducibility of sample applications and densitometric scan of the TZP band was performed in five replicates at a concentration of 50 μg/mL. The obtained RSD values were 0.19% and 0.25%, respectively. Carry-over was assessed by injecting a blank blood sample after the injection of the upper limit of quantification (ULOQ) (100 μg/mL) of the calibration curve. The obtained chromatogram of blank blood sample indicated that the sample carry-over effect was not observed after the injection of ULOQ of the calibration curve.

Table 1 Accuracy and precision data

Actual concentration (μg/mL)	Measured concentration (μg/mL)	Recovery (%)	RSD (%)	Bias (%)
Within-day (n = 5)				
2	1.75	87.52	0.82	−12.5
10	9.01	90.10	0.68	−9.9
50	47.04	94.08	0.37	−5.92
Between-day (n = 15)				
2	1.76	88.02	1.47	−12.0
10	9.05	90.46	0.65	−9.5
50	46.93	93.85	0.39	−6.14
Average		90.67		

Stability of TZP was evaluated at two concentration levels 25 and 100 μg/mL of TZP in spiked blood under different storage conditions: 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 6 h. Percentage stability was expressed as accuracy (%). The results of the stability study have shown that the accuracy was within ± 15% of the nominal value and there was no significant decrease in the concentration of TZP in the stability samples under different storage conditions. In general, OPP compounds tend to decompose faster at higher temperatures in blood [4, 6]. No such decomposition was observed for TZP at room temperature. Many organophosphate pesticides are unstable in blood, because of their degradation by esterase activity [7, 14, 29]. But on the other hand, organophosphorothioate-like TZP and other related compounds are stable in blood and are not metabolized by these enzymes due to the fact that proteins and lipids present in the biological specimens may stabilize these compounds [7, 29]. The results of the stability study were summarized in Table 2. Overall, the measured concentrations of TZP under different storage conditions did not deviate more than 2.69% from the nominal concentration. TZP was found to be stable in the solvent (methanol) used to prepare the solution at room temperature and at 4 °C for periods of 0 h, 3 h, and 6 h. The measured RSD values of peak area were within 8.79%.

Table 2 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)	101.12	1.86	98.91	1.67
Bench-top stability (4 h)	100.12	0.32	100.08	0.12
Long-term stability (1 month)	101.24	2.69	102.19	1.11
Processed sample stability (6 h)	103.00	0.35	102.35	0.05

TZP was also found to be stable (RSD < 1.7% and error < 2.37%) at 0 h and after 3 h and 6 h on the TLC silica gel $60F_{254}$ plates.

3.2 Application of the method to medico-legal cases

Case 1: A person dejected in life over a family issue, attempted suicide by consuming an unknown pesticide and also ingested the same pesticide to his 10-year-old son. After 4 h of treatment, the boy was declared dead. The autopsy was conducted 24 h after the death. An unlabeled pesticide container along with the autopsy samples of the stomach, small intestine, liver, kidney, and blood was sent for toxicological analysis.

Case 2: Under the influence of alcohol, a 21-year-old man ingested an unknown amount of Ghatak® pesticide solution containing 40% EC TZP. He was admitted to the hospital and died after 8 h. The autopsy was carried out 5 h after the occurrence of death. For toxicological investigation, the stomach, small intestine, blood, and portions of the liver and kidney were sent.

A generally unknown screening for pesticides in the autopsy samples was conducted using TLC, TLC–UV densitometry, and GC–MS (GCMS 2020EI, Shimadzu, Japan) methods following LLE. TLC followed by post chromatographic derivatization with palladium chloride reagent indicated the presence of pesticides of organothiophosphate group. Presence of TZP was confirmed by HPTLC–UV scan with the library match and GC–MS electron ionization (EI) full scan screening. A high concentration of TZP was detected in the stomach contents, indicating the oral administration of the pesticide solution. The proposed TLC method was applied for the quantification of TZP in postmortem blood samples of case 1 and case 2, in which 22.01 μg/mL and 27.43 μg/mL were detected respectively. In spite of the rapid metabolism of OPPs either chemically or enzymatically, high levels of parent pesticide can be normally expected in the above-referred two cases, in which large amounts of the pesticide are usually ingested by the deceased persons. Organophosphorus concentrations in human tissues in the range of 10–100 μg/mL reflect acute accidental or intentional exposure [1]. TZP concentrations in postmortem blood samples of the two above-referred cases are within the range of fatal levels, which helped to conclude that the death was caused by intentional exposure. Comparison of our analytical results with others obtained in similar situations or with other reported OPP poisoning cases is difficult due to the 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 the autopsy; and the storage conditions and stability of the analyte in the sample [30, 31]. Determination of the OPP 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 [20, 32, 33]. Since TZP is organothiophosphate-like chlorpyrifos, it 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. Therefore, levels in the blood represent a steady-state concentration [20].

4 Conclusion

The study clearly demonstrated that the obtained values of validation parameters were within the limit of acceptable variability, taking into consideration the analysis of TZP from the whole blood sample. The developed extraction procedure using 1 mL of whole blood has provided the key to the success of method development with excellent extraction yield. The proposed TLC–UV densitometric method has been proven to be simple, rapid, and sensitive for determining TZP in whole blood samples and thus highly suited to the forensic toxicological analysis of TZP, especially in fatal poisoning cases. The reported TZP concentrations in postmortem blood samples were helpful in concluding the cause of death for medical

officers and also offer useful information to forensic toxicologists in the interpretation of forensic results. This is the first validated procedure for determining TZP in human whole blood samples to the best of our knowledge.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals.

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