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TECHNICAL NOTE

TOXICOLOGY

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Development and Validation of a Simple GC–MS Method for the Simultaneous Determination of 11 Anticholinesterase Pesticides in Blood—Clinical and Forensic Toxicology Applications

ABSTRACT: Anticholinesterase pesticides are widely used, and as a result they are involved in numerous acute and even fatal poisonings. The aim of this study was the development, optimization, and validation of a simple, rapid, specific, and sensitive gas chromatography–mass spectrometry method for the determination of 11 anticholinesterase pesticides (aldicarb, azinphos methyl, carbofuran, chlorpyrifos, dialifos, diazinon, malathion, methamidophos, methidathion, methomyl, and terbufos) in blood. Only 500 μL of blood was used, and the recoveries after liquid–liquid extraction (toluene/chloroform, 4:1, v/v) were more than 65.6%. The calibration curves were linear ($R^2 \geq 0.996$). Limit of detections and limit of quantifications were found to be between 1.00–10.0 and 3.00–30.0 μg/L, respectively. Accuracy expressed as the % E_r was found to be between −11.0 and 7.8%. Precision expressed as the percent relative standard deviation was found to be <9.4%. The developed method can be applied for the investigation of both forensic and clinical cases of accidental or suicidal poisoning with these pesticides.

KEYWORDS: forensic science, anticholinesterase pesticides, blood, gas chromatography/mass spectrometry, liquid–liquid extraction, clinical and forensic toxicology

Pesticides are chemical substances used in most countries around the world to protect agricultural and horticultural crops by preventing, controlling, or lessening the damage caused by pests (1–3). Although there are obvious benefits from the use of pesticides, there are also significant problems because of their use or misuse such as environmental contamination, occupational exposure of farmers and accidental exposure of bystanders, and potential toxicity to animals and to humans owing to accidental or intentional exposure (4–6). Anticholinesterase pesticides are widely used at home and at work to ensure a pest-free environment, and as a result, they can cause numerous acute, subacute, or chronic, and sometimes fatal, poisonings that could occur accidentally or after a suicidal attempt (7–10). At home, poisoning may occur by oral ingestion (voluntary or not), whereas occupational users most frequently encounter dermal exposure or inhalation (1,11). Therefore, exposure to anticholinesterase pesticides should always be taken into consideration when investigating health problems especially to agricultural populations (12–14). In such cases, blood samples may be sent to a toxicological laboratory either for the diagnosis of a poisoning, within the frame of emergency toxicology, or for the determination of the cause of death in fatal cases within the frame of forensic toxicology.

The mechanism of toxicity of all anticholinesterase pesticides is based on their anticholinesterase properties and mainly on the inhibition of acetylcholinesterase because of direct enzyme binding, resulting in the accumulation of acetylcholine at the nerve synapses. The levels of the activity of plasma pseudocholinesterase and red blood cell (RBC) cholinesterase reflect the effect of the absorbed pesticides on this enzymatic system of the organism (15,16). The inhibition of cholinesterases leads to muscarinic, nicotinic, and central nervous system (CNS) symptoms. The muscarinic and nicotinic symptoms observed after poisoning with such anticholinesterase agents include diarrhea, urination, miosis, bronchospasm, muscular twitching, emesis, lacrimation, and salivation (7,17). Owing to their CNS action, convulsions appear, sometimes tonic (limbs stretched and rigid), but they are more likely to be clonic (rapid repetitive movements) in nature (17,18). In fatal cases, the cholinesterase inhibition leads to the disruption of specific nerve functions (centrally and peripherally), convulsions, respiratory failure, and when untreated, anoxia (7,18).

In clinical practice, the quick diagnosis of an anticholinesterase poisoning is based on the measurement of plasma cholinesterase activity while the determination of the pesticide responsible confirms the exposure. In fatal cases, where the postmortem measurement of the enzyme activity is not reliable, the determination of the pesticide responsible in the biological fluids of the deceased can be helpful in establishing pesticide poisoning as the cause of death (17,19,20).

Most of the previously published methods that determine simultaneously pesticides or their metabolites in biological samples (blood, serum, plasma, urine, hair, and breast milk) employ

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high-performance liquid chromatography (HPLC) (20,21), gas chromatography–nitrogen phosphorous detector (GC-NPD) (17), gas chromatography–mass spectrometry (GC–MS) (17,22–27) or gas chromatography tandem mass spectrometry (GC–MS/MS) (28–31), and liquid chromatography–mass spectrometry (LC–MS) (32–34), or liquid chromatography tandem mass spectrometry (LC–MS/MS) (35). Some of these methods use expensive equipment and laborious and/or time-consuming extraction or derivatization procedures that are not useful in emergency cases of severe poisoning. Hence, the development of rapid, selective, and reliable methods, allowing the identification and quantification of as many pesticides as possible in human biofluids, is extremely important for emergency toxicology (1). The aim of this study was the development, optimization, and validation of a GC–MS method for the determination of 11 anticholinesterase pesticides (aldicarb, azinphos methyl, carbofuran, chlorpyrifos, dialifos, diazinon, malathion, methamidophos, methidathion, methomyl, and terbufos) in whole blood. These pesticides were selected owing to their significant toxicity and their extensive market presence in Greece. Whole blood is the most appropriate matrix for estimating toxic effect (27). It has to be mentioned here that analysis of serum/plasma samples may not indicate correctly the total concentrations because of plausible strong binding of pesticides at erythrocytes (36). Therefore, development of analytical methods using a small volume of whole blood, instead of serum or plasma, is preferred. The developed method is simple, rapid, and accurate, and it also has the advantage of using only a small volume of blood (0.5 mL) for the identification and the determination of the above-mentioned pesticides. This method can also be used by emergency toxicology units for the differential diagnosis of poisoning with pesticides and by forensic laboratories for the establishment of a cause of death. Examples of intoxication cases investigated, using the proposed method, are reported herein to demonstrate the suitability of the method.

Methods

Chemicals and Reagents

Reference standards of the 12 anticholinesterase pesticides (aldicarb 99.9%, methomyl 99.9%, methamidophos 99.1%, carbofuran 99.9%, terbufos 98.6%, diazinon 99.0%, malathion 97.3%, chlorpyrifos 99.2%, methidathion 96.0%, azinphos methyl 98.5%, dialifos 99.6%, and mevinphos 98.6%) were purchased from Sigma-Aldrich (Seelze, Germany). The above-mentioned purities and the storage conditions were according to their commercial certificates. The solvents used (hexane, ethyl acetate [EtAc], dichloromethane, chloroform, toluene, and isopropanol) were of analytical or HPLC grade and were purchased from Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA; disodium salt) was of analytical grade and was obtained from Serva (Heildelberg, Germany). Nexus (Varian, Houten, Netherlands) solid-phase extraction (SPE) columns were used. Human blood was obtained, after informed consent, from healthy donors, and before its use, it was screened by GC–MS for the presence of pesticides. EDTA was added to blood up to a final concentration of 10 mg/mL before spiking with working standard solutions as it prevents the loss of the organophosphates (17).

GC–MS Analysis and Other Apparatus

GC–MS analysis of the 11 pesticides was performed on a Hewlett-Packard GC 5890 Series II interfaced with an HP 5970 Series mass selective detector (MSD) and equipped with an automatic injector HP7673A (Hewlett Packard, Rockville, MD). The separation of analytes was carried out using a cross-linked HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). Helium was used as carrier gas at a flow rate of 1.1 mL/min. The temperatures of injection port and interface were 250 and 300°C, respectively. Initial oven temperature of 60°C was held for 5 min, followed by an increase to 170°C (hold for 5 min) at a rate of 15°C/min, 220°C (0 min hold) at a rate of 5°C/min, and 300°C (hold for 3 min) at a rate of 25°C/min. The MSD was operated at electron impact ionization mode (EI, 70 eV), and the mass range was 50–600 amu. The three major ions of each pesticide were selected from the mass spectra obtained from standard reference materials, and they were used in SIM acquisition mode (dwell time of 10 msec) for the identification and quantification of each analyte. The confirmation ions and the retention times for each analyte are presented in Table 1 (the quantification ion is in boldface).

A 691 digital pH meter (Metrohm, Herisau, Switzerland) with a glass electrode was used for pH adjustments. A vortex (Model MT 19; Chiltern, London, UK), an evaporating device using nitrogen (Model 18780, Reacti-Vap; Pierce, Rockford, IL), and a cooling centrifuge (4K10; Sigma, Osterade, Germany) were also used. Purified water was obtained from a Direct-Q water purification system of Millipore (Billerica, MA).

Preparation of Standard Solutions

Stock standard solutions of each pesticide at a concentration of 10.0 mg/mL were prepared by dissolving the appropriate amount of each pesticide in toluene. These solutions were stored at −20°C and used for the preparation of the relative working standard solutions. Calibration and quality control (QC) blood samples were prepared by spiking 450 μL of blank blood with 50 μL of the relative working standard solutions containing the 11 compounds at the following concentrations: 0.03, 0.09, 0.1, 0.2, 0.3, 0.5, 1.0, 1.5, and 2.0 μg/mL for chlorpyrifos, diazinon, malathion, methidathion, and terbufos; 0.15, 0.45, 0.5, 1.0, 1.5, 2.5, 5.0, 7.5, and 10.0 μg/mL for carbofuran and dialifos; 0.3, 0.9, 1.0, 2.0, 3.0, 5.0, 10.0, 15.0, and 20.0 μg/mL for aldicarb, azinphos methyl, methamidophos, and methomyl. Internal standard working solution of mevinphos at a concentration of 2.0 μg/mL was prepared from the corresponding stock solution (10.0 mg/mL).

Extraction Procedure

In all samples derived from clinical or forensic cases, EDTA was added up to a final concentration of 10 mg/mL, and the blood samples were stored at −20°C until their analysis (in <48 h).

TABLE 1—*Monitored ions and retention time for each pesticide. The quantification ion is in boldface.*

Pesticide	Monitored Ions	Retention Time (min)
Aldicarb	**86**, 58, 144	8.80
Azinphos methyl	**160**, 132, 93	30.47
Carbofuran	**164**, 149, 221	17.82
Chlorpyrifos	**197**, 314, 258	23.49
Dialifos	**208**, 357, 186	30.95
Diazinon	**179**, 137, 304	19.23
Malathion	**125**, 173, 158	23.11
Methamidophos	**94**, 95, 141	11.59
Methidathion	**145**, 125, 302	25.77
Methomyl	**58**, 105, 88	10.83
Terbufos	**231**, 153, 288	18.67
Mevinphos (IS)	**127**, 192, 164	14.51

Internal standard solution (50 μL of 2.0 μg/mL mevinphos) was added to 500 μL of blood, and the tubes were vortex-mixed for 1 min. The blood samples underwent liquid–liquid extraction (LLE) (without any pH adjustment) with 1.5 mL of solvent mixture of toluene and chloroform (4:1, v/v), by intensive vortexing for 5 min. After centrifuging at 16,250 × g for 10 min at 4°C, the organic phase was transferred into a clean tube and evaporated to dryness under a gentle stream of N_2 at 40°C, and 50 μL of hexane was added to reconstitute the residues. Chromatograms were obtained with the injection of 1 μL of hexane onto the GC–MSD system.

Validation Data

For the validation of the GC–MS method, the following criteria were used (according to FDA [37] and ICH [38] guidelines): selectivity, limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy, absolute recovery, specificity, robustness, and stability. Selectivity, linearity, precision, and accuracy of the method were validated through five analytical runs on five different days. The way each parameter was determined can be found in the Results and Discussion section.

Results and Discussion

Method Development and Optimization

A GC–MS method has been developed and optimized to determine the concentrations of 11 pesticides in blood samples. The isolation of these analytes from the specimens was achieved after LLE.

To optimize the chromatographic separation of the 11 pesticides studied, different oven programs were tested. Chromatographic conditions, including injector, interface, and initial and final column temperatures, as well as column temperature rate and carrier gas flow rate, were optimized.

During the optimization of the extraction procedure, LLE of blood samples was tested using many systems of organic solvents, as well as SPE with nonconditioned SPE columns (Nexus). The SPE procedure included a washing step with water, and after drying at high vacuum (≥10 mmHg) for 5 min, the elution of pesticides was achieved by EtAc. In SPE, the recoveries of azinphos methyl, dialifos, methamidophos, methidathion, and methomyl were found to be extremely low (<20%) and therefore proved to be an ineffective procedure. During LLE, a variety of organic solvents or mixtures of them—toluene, EtAc, chloroform, hexane/dichloromethane (3:1, v/v, mixture 1), toluene/EtAc (3:2, v/v, mixture 2), toluene/isopropanol (9:1, v/v, mixture 3), toluene/chloroform (4:1 and 1:1, v/v, mixtures 4 and 5, respectively), toluene/dichloromethane (9:1, v/v, mixture 6), and toluene/chloroform/isopropanol (4:4:2, 5:4:1, 6:3:1, 8:2:1, and 5:4:0.5, v/v/v, mixtures 7, 8, 9, 10, and 11, respectively)—were tested. According to the recoveries of the 11 pesticides (Table 2), mixture 4 (toluene/chloroform 4:1, v/v) was the optimum (bold values in Table 2) for the extraction procedure. Different extraction pHs were also checked (pH 5–9), but finally no addition of a buffer was decided based on the results of the recovery for all analytes of interest. This final extraction procedure led to high selectivity and extraction efficiency for the 11 pesticides, without interferences from endogenous compounds of blood. A representative chromatogram obtained from an extracted spiked blood sample at the low QC concentration for each pesticide is presented in Fig. 1 (9.00 μg/L for chlorpyrifos, diazinon, malathion, methidathion, and terbufos; 45.0 μg/L for carbofuran and dialifos; 90.0 μg/L for aldicarb, azinphos methyl, methamidophos, and methomyl).

In comparison with the previously published method of Tarbah et al. (17), the developed method is suitable for the determination of not only organophosphates but also the most important carbamates, at least of the ones used in Greece (aldicarb, carbofuran, methomyl). The LLE with a mixture of toluene and chloroform (4:1, v/v) instead of only toluene (17) led to higher recovery results especially for azinphos methyl and methamidophos (Table 2).

Method Validation

Selectivity was studied by analyzing six different blank samples, and the matrix effect was assessed. All blank blood samples were free of co-eluting peaks at the retention times of the analytes of interest. The selectivity of the method was adequate with minimal matrix effect in all blank samples.

Specificity was determined by analyzing a standard mixture of commonly used drugs as well as their metabolites (diazepam, nordiazepam, bromazepam, alprazolam, 7-amino-flunitrazepam, paracetamol, phenobarbital, amitriptyline, clomipramine, biperiden, carbamazepine, citalopram, clozapine, paroxetine, olanzapine, venlafaxine, thioridazine, zolpidem, haloperidol, mirtazapine, levomepromazine, phenytoin, and valproic acid) at a concentration of 10 mg/L. These drugs can be used by people occupationally or accidentally exposed to pesticides and consequently could interfere with their determination. Spiked human blood samples (n = 6) with these substances at a concentration of 1 mg/L were also analyzed. The specificity study documented that blood concentrations of 1 mg/L of the drugs selected do not interfere with the accurate determination of the included 11 pesticides in human blood.

TABLE 2—*Absolute recovery of the 11 pesticides using LLE with organic solvents and mixtures of them.*

	% Absolute Recovery													
Pesticide	Toluene	EtAc	Chloroform	Mix 1	Mix 2	Mix 3	Mix 4	Mix 5	Mix 6	Mix 7	Mix 8	Mix 9	Mix 10	Mix 11
Aldicarb	97.3	105.0	74.2	135.3	79.3	73.2	**98.6**	75.7	90.9	71.0	86.5	101.0	78.7	76.0
Azinphos methyl	39.9	87.0	52.5	41.7	63.6	42.1	**109.8**	77.8	145.9	22.6	34.7	53.2	31.7	85.0
Carbofuran	106.1	111.6	72.5	146.1	85.9	76.0	**99.1**	76.5	95.0	94.0	97.2	108.0	85.6	82.6
Chlorpyrifos	91.8	75.6	67.0	38.6	73.2	72.0	**108.2**	90.1	87.3	110.0	101.9	118.1	92.9	81.7
Dialifos	106.7	139.0	100.0	30.5	43.1	32.4	**86.7**	60.7	48.1	42.4	71.8	132.8	104.3	113.9
Diazinon	104.9	86.4	79.7	36.9	86.6	83.0	**107.2**	83.7	92.5	89.0	99.4	105.0	83.2	82.2
Malathion	95.4	92.0	73.1	56.4	79.0	70.5	**107.6**	89.5	96.1	87.0	93.8	108.0	81.2	86.8
Methamidophos	48.3	0.0	37.6	3.0	16.1	13.6	**67.9**	18.8	8.7	119.0	70.9	47.0	26.9	15.0
Methidathion	101.8	101.0	74.0	64.7	73.0	62.2	**101.4**	83.0	86.6	68.8	81.0	104.4	74.8	83.7
Methomyl	72.1	81.0	73.1	117.5	63.8	40.0	**92.2**	66.4	68.4	114.0	76.4	95.0	75.7	75.0
Terbufos	94.1	67.0	69.4	37.2	73.6	75.4	**88.4**	69.3	78.1	87.0	91.4	93.6	74.6	76.3

Boldface showing Mix 4 is optimum for the extraction procedure.

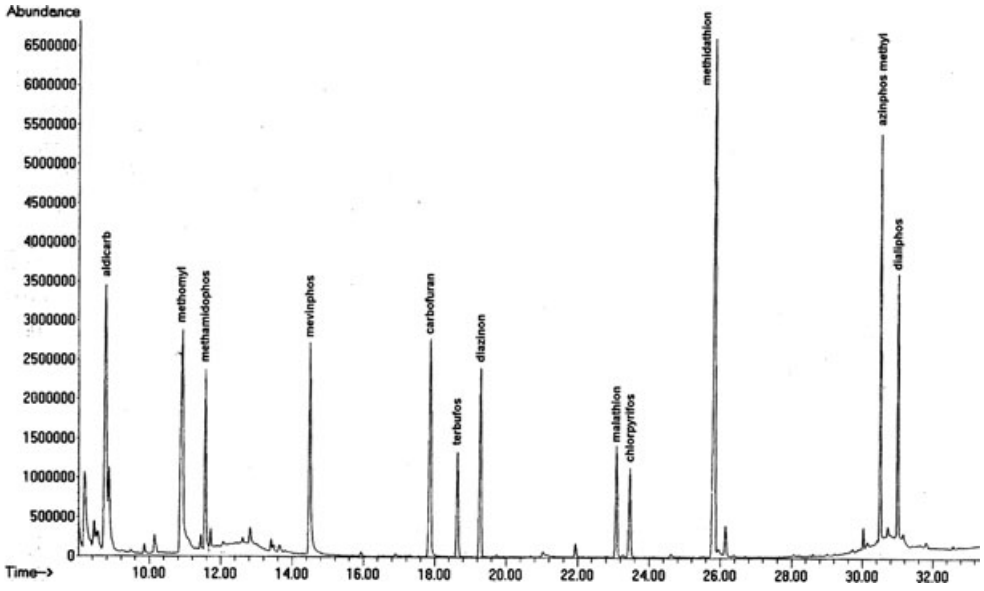

FIG. 1—*Chromatogram of a spiked blood sample of 11 pesticides at the low QC concentration and their internal standard (mevinphos).*

The LOD and LOQ for each pesticide were determined as the lowest concentration yielding signal-to-noise ratios of at least 3:1 and 10:1, respectively, with correct relative ion intensities and a retention time within ±0.2 min of the average calibrator retention time. LODs and LOQs for the 11 pesticides were found to be between 1.00–10.0 and 3.00–30.0 μg/L, respectively, and are presented in Table 3. The LOQ for each pesticide was defined as the lowest concentration at which precision and accuracy, expressed by relative standard deviation (RSD), were lower than 20%.

Linearity was determined by the calculation of the regression line using the method of least squares with a weighting factor of $1/x^2$, and it was expressed by the correlation coefficient (R^2). Calibration curves of spiked calibrator blood samples showed excellent linearity, for all analytes in five different runs. The linear dynamic range was 3.00–200.0 μg/L for terbufos, diazinon, malathion, chlorpyrifos, and methidathion; 15.0–1000.0 μg/L for carbofuran and dialifos; and 30.0–2000.0 μg/L for aldicarb, methomyl, azinphos methyl, and methamidophos, with correlation coefficients (R^2) exceeding 0.996. Linearity results are shown in Table 3.

Absolute recovery for QC samples was assessed by running five replicates at the relative low and high concentrations and by eight replicates at the medium concentration. Absolute recovery of the method was calculated as the percentage of the response of each analyte in the sample compared with that of a standard solution containing the analyte at the same concentration. The following equation was used: % absolute recovery = (peak area of spiked blood sample) × 100/(mean peak area of 6 runs of standard solution).

The absolute recovery (calculated at three QC levels in five different runs) for each pesticide was found to be higher than 65.6% and is presented in Table 3.

Precision and accuracy of the method (intraday, $n = 6$ and inter-day, $n = 30$) were calculated by analyzing three QC levels (see Table 4) within the linear range of an analyte. The concentration of the QC samples was calculated by the calibration curve of each day of analysis. Precision was expressed as the relative standard deviation (% RSD). Accuracy of the method was calculated as the percent difference from the expected concentration (% E_r). Intra- and interday accuracy was found to be between −8.7% to 7.8% and −11.0% to 6.2%, respectively, while intra- and interday precision had % RSDs <6.1% and 9.4%, correspondingly, for all analytes (Table 4).

Robustness of the entire method was studied by changing several parameters of the procedure (the volume of the extraction mixture,

TABLE 3—*Linearity results, limit of detection (LOD), limit of quantification (LOQ), and % absolute recovery of the developed method for the 11 pesticides (five different runs).*

Pesticide	$y = a(\pm s_a)x + b(\pm s_b)$	R^2	% RSD of Slopes ($n = 5$)	LOD (μg/L) S/N 3:1	LOQ (μg/L) S/N 10:1	% Absolute Recovery
Aldicarb	$y = 0.0012(\pm0.0001)x + 0.0273(\pm0.0313)$	≥0.998	6.9	10.0	30.0	92.4–102.3
Azinphos methyl	$y = 0.0040(\pm0.0002)x - 0.1915(\pm0.1280)$	≥0.997	4.1	10.0	30.0	95.1–110.0
Carbofuran	$y = 0.0063(\pm0.0003)x + 0.1248(\pm0.2286)$	≥0.999	4.3	5.00	15.0	88.9–103.1
Chlorpyrifos	$y = 0.0151(\pm0.0008)x + 0.0811(\pm0.0342)$	≥0.996	5.4	1.00	3.00	96.7–108.9
Dialifos	$y = 0.0030(\pm0.0003)x - 0.0061(\pm0.0775)$	≥0.994	9.9	5.00	15.0	79.1–88.9
Diazinon	$y = 0.0192(\pm0.0011)x + 0.1155(\pm0.0405)$	≥0.996	5.7	1.00	3.00	95.3–109.4
Malathion	$y = 0.0129(\pm0.0009)x + 0.0766(\pm0.0301)$	≥0.996	6.8	1.00	3.00	92.3–108.4
Methamidophos	$y = 0.0010(\pm0.0001)x - 0.0248(\pm0.0442)$	≥0.994	12.9	10.0	30.0	65.6–72.8
Methidathion	$y = 0.0218(\pm0.0008)x - 0.0786(\pm0.1999)$	≥0.997	3.9	1.00	3.00	91.1–102.0
Methomyl	$y = 0.0012(\pm0.0002)x - 0.0323(\pm0.0541)$	≥0.996	14.4	10.0	30.0	85.6–93.5
Terbufos	$y = 0.0205(\pm0.0010)x + 0.1236(\pm0.0341)$	≥0.994	4.8	1.00	3.00	79.8–90.2

RSD, relative standard deviation.

TABLE 4—*Accuracy and precision at three quality control levels for the determination of the 11 pesticides in blood.*

		Intraday (n = 6)		Interday (n = 30)	
Pesticide	Quality Control Levels (μg/L)	Accuracy (% E_r)	Precision (% RSD)	Accuracy (% E_r)	Precision (% RSD)
Aldicarb	90.0/300.0/1500.0	−4.9/3.1/7.8	0.3/0.5/1.0	−4.3/2.0/1.5	4.4/6.9/4.3
Azinphos methyl	90.0/300.0/1500.0	3.9/−4.4/3.5	2.1/1.1/0.5	3.0/−5.1/0.8	0.8/4.1/4.7
Carbofuran	45.0/150.0/750.0	−4.4/5.0/−3.6	0.3/2.4/1.5	−7.4/−0.2/1.2	5.4/7.6/3.8
Chlorpyrifos	9.00/30.0/150.0	2.4/5.7/−0.6	1.1/1.3/0.6	−0.2/3.3/2.0	7.2/6.1/2.4
Dialifos	45.0/150.0/750.0	−7.7/−3.6/2.0	2.6/2.9/0.1	−7.6/−2.0/−0.1	7.2/9.4/5.6
Diazinon	9.00/30.0/150.0	−2.9/−2.6/−7.0	3.6/2.2/1.8	0.7/3.3/−0.1	8.4/3.8/4.1
Malathion	9.00/30.0/150.0	−2.2/−5.6/4.2	0.1/0.2/0.6	−6.1/2.8/1.6	5.6/5.5/1.6
Methamidophos	90.0/300.0/1500.0	3.0/−3.9/−6.4	1.7/2.4/0.9	0.8/1.3/−1.8	8.7/5.2/4.8
Methidathion	9.00/30.0/150.0	−2.1/−3.9/3.2	1.2/0.1/0.2	0.3/−0.3/1.3	2.4/5.8/2.2
Methomyl	90.0/300.0/1500.0	−8.7/−6.2/4.7	6.1/1.3/3.1	−11.0/−1.2/−0.1	4.3/6.1/6.4
Terbufos	9.00/30.0/150.0	−2.2/2.3/5.7	0.7/1.7/3.6	3.0/3.3/6.2	5.0/4.0/2.1

RSD, relative standard deviation.

1.4 and 1.6 mL instead of 1.5 mL, and the ratio of the solvents in the extraction mixture, 4.5:1 and 3.5:1, v/v instead of 4:1, v/v), as well as chromatographic parameters (initial column temperature, 55 and 65°C instead of 60°C, and injector temperature, 245 and 255°C instead of 250°C). The differences between the mean area of each analyte, for each parameter of the method changed, as well as their standard deviation were calculated. Neither a single parameter nor a combination of the ones changed showed a significant influence on the results of the method, which proved to be sufficiently robust.

The stability of pesticides was assessed by analyzing spiked human blood with the relative analytes at low and high QC concentrations and keeping the samples at 4°C for 3 days (72 h) and −20°C for 1 week. Furthermore, frozen spiked human blood samples were subjected to three freeze–thaw cycles. EDTA was added to blood up to a final concentration of 10 mg/mL before spiking with working standard solutions. The loss for 11 pesticides (at low and high QC concentrations) was found to be between 11.3 (diazinon) and 45.1% (methamidophos) at 4°C for 3 days, between 9.4 (methidathion) and 33.2% (carbofuran) at −20°C for 1 week, and between 4.6 (malathion) and 14.0% (aldicarb) at three freeze–thaw cycles. It has to be mentioned here that pesticides generally decompose in the blood (39). The most unstable pesticides under the storage conditions of our study proved to be methamidophos, malathion, and carbofuran. In cases of lethal pesticide poisonings, it should be kept in mind that the blood concentrations of relative compounds might decrease during the postmortem interval. Therefore, the concentrations that will be found can be considerably lower at the time of analysis than the ones at the time of death.

Method Application

The developed method for the simultaneous determination of 11 pesticides in blood was successfully applied by our laboratory to the investigation of forensic and/or clinical toxicological cases of accidental and suicidal poisoning related to pesticide ingestion. The determination of the pesticides was accomplished by GC–MSD using the developed method. Examples of investigated intoxication cases, using the proposed method, are presented below to demonstrate its suitability.

Case 1—A 38-year-old male farmer, during field chemical pest control, started feeling unwell showing symptoms of sedation, acute abdominal pain, and gradual loss of consciousness. The patient was immediately admitted to a hospital where it was found that he had depressed RBC cholinesterase and plasma pseudocholinesterase activity. Toxicological analysis revealed the presence of methamidophos in venous blood at a concentration of 778 μg/L. Antidotal treatment (pralidoxime and atropine) was given. Normal levels of pseudocholinesterase were regained, and the man was discharged after a total of 21 days of hospitalization. This case was a typical case of pesticide poisoning after occupational exposure.

Case 2—A 62-year-old man presented with sudden onset of vomiting, abdominal pain, loss of balance, and hypersalivation. The patient was immediately admitted to a hospital where it was found that the levels of his plasma pseudocholinesterase were extremely low, without any other laboratory findings. In a venous blood sample that was sent to the toxicology unit, methomyl was identified and was determined at a concentration of 612 μg/L. During the investigation of the case, the police ascertained that his wife, after a fight with her husband, had added methomyl into his coffee with the intention to murder him. The man fully recovered after the appropriate treatment (mainly atropine), and after hospitalization for 10 days, he was discharged without any clinical sequelae. This case is representative of a poisoning with pesticide after a homicide attempt.

Case 3—A 27-year-old man was found dead at an abandoned house in the countryside. The man had a history of psychological problems in the past, and a suicide letter was also found close to him. During autopsy, only extensive pulmonary edema was found. Analysis of his stomach content showed the presence of carbofuran, while the analysis of a femoral blood sample revealed the presence of carbofuran at a concentration of 466 μg/L. According to the case history and the toxicological analysis, this is a representative case of a fatal suicide attempt.

Case 4—A 2-year-old child was admitted to a children's hospital with symptoms of drowsiness, vomiting, abdominal pain, dysarthria, and loss of balance. His plasma pseudocholinesterase levels were extremely low. A toxicological analysis was performed within the frame of differential diagnosis and showed that his symptoms were not because of pathological reasons, but the result of poisoning. Presence of carbofuran was confirmed in a venous blood sample at a concentration of 91 μg/L. Investigation showed that the child, about 3 h before his admission, had ingested grains of carbofuran from the flower pots of his balcony. After hospitalization for a week, the levels of plasma pseudocholinesterase returned to normal, and the child was discharged. This case is a typical case of an accidental poisoning.

Conclusions

Pesticides are used extensively worldwide, therefore, they could be the cause of either intentional or unintentional poisonings, fatal or not. This is of special concern in agricultural areas like Greece. The use of pesticides in suicidal poisonings is common. Moreover, pesticides are often the cause of accidental or occupational poisonings, while these substances are used less frequently for homicidal purposes (9–11,15,16,18,20,24).

Development and validation of a simple, rapid, sensitive, accurate, and specific GC–MS method, using relatively low-cost instrumentation, for the simultaneous determination of anticholinesterase pesticides in blood or other biological fluids are necessary for a toxicological laboratory, either for the diagnosis of poisoning within the frame of emergency toxicology or for the determination of the cause of death within the frame of forensic toxicology. The developed method could contribute to the investigation of both forensic and clinical toxicological cases concerning accidental, suicidal, or homicidal poisonings.

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