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Short communication

Liquid chromatography–tandem mass spectrometry method for the simultaneous quantitative determination of the organophosphorus pesticides dimethoate, fenthion, diazinon and chlorpyrifos in human blood

Paul Salm [a,*], Paul J. Taylor [a,b], Darren Roberts [c], Janaka de Silva [d]

[a] Australian Bioanalytical Services Pty Ltd., Princess Alexandra Hospital, Woolloongabba, Queensland 4102, Australia
[b] Clinical Pharmacology Mass Spectrometry Facility, Princess Alexandra Hospital, Woolloongabba, Queensland 4102, Australia
[c] South Asian Clinical Toxicology Research Collaboration, Faculty of Medicine, University of Peradeniya, Peradeniya, Sri Lanka
[d] Faculty of Medicine, University of Kelaniya, Ragama, Sri Lanka

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ABSTRACT

Simultaneous determination of the organophosphorus pesticides dimethoate, fenthion, diazinon and chlorpyrifos in human blood by HPLC–tandem mass spectrometry was developed and validated. The pesticides were extracted by a simple one-step protein precipitation procedure. Chromatography was performed on a Luna C_{18} (30 mm × 2.0 mm, 3 μm) column, using a step-gradient at a flow rate of 0.4 ml/min. The assay was linear from 0.5 to 100 ng/ml ($r^2 > 0.992$, $n = 24$) for all pesticides. The inter- and intra-day accuracy and precision for the method was 96.6–106.1% and <10%, respectively. The lower limit of quantification was 0.5 ng/ml. In conclusion, the method described displays analytical performance characteristics that are suitable for the quantification of these pesticides in cases of acute poisoning.

1. Introduction

Organophosphorus pesticides (OPs) are amongst the most commonly used pesticide classes worldwide. Unfortunately, they are also important causes of morbidity and mortality following intentional self-poisoning or in cases of occupational or environmental exposure [1,2]. In Sri Lanka, self-poisoning is a major problem, [3] with nearly 50% of pesticide poisonings being due to OPs [4]. The most commonly used OPs in Sri Lanka are dimethoate, fenthion, diazinon and chlorpyrifos.

Upon entering the body, OPs are rapidly metabolized to non-specific and specific metabolites. Measurement of non-specific dialkyl phosphate metabolites in urine has been previously reported and used as an indication of OP exposure [5–11]. However, since many OPs are converted to these non-specific metabolites, identification of a particular parent OP cannot be established. Alternatively, the specific metabolites of diazinon (2-isopropyl-4-methyl-6-hydroxypyrimidine, IMPY) and chlorpyrifos (3,5,6-trichloro-2-pyridinol, TCP) offer useful biomarkers to evaluate systemic exposure to their respective parent compounds [12]. Sensitive analytical methods are required to quantify these compounds in biological fluids.

Measurement of pesticides in biological fluids can reflect exposures from a variety of sources including dermal absorption, ingestion and inhalation [13]. In cases of acute poisoning, the parent compound can be directly monitored in blood providing more accurate information with respect to identifying the parent pesticide, the absorbed dose and the degree of exposure to target tissues prior to elimination from the body [14,15]. However in cases of environmental exposure, parent pesticide concentrations in blood are typically very low and difficult to detect. Therefore the measurement of urinary metabolites, which are usually an order of magnitude greater in concentration, would be considered the most appropriate approach in environmental exposure studies [11].

Several reviews have documented the progression of analytical methods for the biological monitoring of OPs and their metabolites [16–18]. The traditional high-performance liquid chromatography (HPLC), gas chromatography (GC) and GC–single stage mass spectrometry (MS) based methods were typically burdened with complex labor intensive sample extraction procedures that can include derivatization. Progression to more sensitive and selective tandem mass spectrometry techniques has led to simplified

* Corresponding author at: Australian Bioanalytical Services Pty Ltd., Level 3, R-Wing, Building 1, Princess Alexandra Hospital, Ipswich Road, Woolloongabba, Brisbane, Queensland 4102, Australia. Tel.: +61 7 3240 5334; fax: +61 7 3240 5031.
E-mail address: psalm@austbioservices.com.au (P. Salm).

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sample preparation and lower limits of detection, which is necessary to confirm significant absorption in low-grade exposures. Several articles have reported methods for the measurement of these selected OP parent pesticides in blood, serum or plasma [14,19–24]. All of the HPLC, GC–MS or GC–tandem mass spectrometry (MS/MS) methods are based on liquid–liquid and solid phase extraction prior to analysis. However, a HPLC–tandem mass spectrometry (HPLC–MS/MS) method by Sancho et al. [21] utilized protein precipitation to measure chlorpyrifos and its major metabolite with a two-dimensional chromatography technique that requires a column switching system with two separation columns and an additional HPLC pump. Similarly, a HPLC–MS/MS procedure by Inoue et al. [24] used protein precipitation followed by supernatant filtration (0.45 μm filter) to determine several OPs. However, this method can only be applied to cases of severe acute poisoning as the lower limit of quantification for fenthion (1250 ng/ml) and diazinon (250 ng/ml) may not be sufficiently sensitive to measure moderate to low acute pesticide exposure.

The aim of this study was to develop and validate a HPLC–MS/MS method with a simple one-step sample precipitation procedure, to simultaneously measure the OPs dimethoate, fenthion, diazinon and chlorpyrifos in blood. The method was applied to a case of acute intentional diazinon self-poisoning with confirmation using a secondary mass transition.

2. Experimental

2.1. Chemicals

Analytical standard solutions (Pestanal®, Riedel-de Haën) of dimethoate, fenthion, diazinon and chlorpyrifos (100 mg/ml) and chlorpyrifos-diethyl-d_{10} were sourced from Sigma–Aldrich (Castle Hill, NSW, Australia). Dimethoate-dimethyl-d_6, fenthion-dimethyl-d_6 and diazinon-diethyl-d_{10} were purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). HPLC grade methanol was purchased from Merck KGaA (Darmstadt, Germany). Ammonium acetate, formic acid and zinc sulphate were supplied from Sigma Chemical Company (St Louise, MO, USA). Deionized water was obtained from a Milli-Q water purification system (Millipore, Milford, MA, USA).

Stock solutions of pesticides (1 μg/ml) and internal standards chlorpyrifos-diethyl-d_{10} (500 μg/ml), dimethoate-dimethyl-d_6 (100 ng/ml), fenthion-dimethyl-d_6 (5 μg/ml) and diazinon-diethyl-d_{10} (1 μg/ml) were prepared in methanol and stored at −20 °C in the dark. Calibration and quality control material were prepared in-house using whole blood. Calibration samples were prepared at concentrations of 0.5, 1.0, 5.0, 10, 25, 50, 100 ng/ml and quality control samples were prepared at 0.5, 2.5, 20, 75, 100, 250 and 2500 ng/ml. The quality control samples at 250 and 2500 ng/ml were used for the dilution suitability study.

2.2. HPLC–mass spectrometry apparatus and conditions

Chromatography was performed on an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany). Extracted samples were stored in amber glass vials at 4 °C in the autosampler tray until analysis. To minimize sample carry-over effects, a programmed injector wash function utilizing five consecutive needle washes (methanol:deionized water, 50:50, v/v) was used. The HPLC analytical column was a Luna C_{18} reverse phase column (30 mm × 2.0 mm i.d., 3 μm, Phenomenex, Lane Cove, NSW, Australia) at 25 °C, using a binary step-gradient at a flow rate of 0.4 ml/min with (A) 2 mmol/l ammonium acetate buffer containing 1 ml/l formic acid in water (pH 2.8) and (B) 2 mmol/l ammonium acetate buffer containing 1 ml/l formic acid in methanol as the mobile phases. The programmed gradient commenced at 40% (B) followed by a direct switch to 80% (B) at 1.5 min followed by a direct switch back to 40% (B) at 6 min. The total chromatographic analysis time for all analytes was 8 min per sample.

Mass spectrometric detection was performed on a linear ion trap quadrupole tandem mass spectrometer (4000QTrap™, Applied Biosystems/MDS Sciex Instruments, Concord, Ontario, Canada) using selected reaction monitoring. Ions were generated in positive ionization mode using an electrospray interface. The source temperature was 500 °C and the source voltage was 5000 V. The nebuliser gas (GS1) and the turbo gas (GS2) was 50 psi. Acquisition was performed over three time periods for each sample. Mass spectrometer quantification and qualification parameters and different compound dependent settings of declustering potential and collision energy for each pesticide are shown in Table 1. Under these conditions the most abundant ions were selected. Peak area ratios obtained from selected reaction monitoring of the mass transitions for each pesticide and their respective internal standards were used for quantification. Data were collected and analyzed using Analyst™ software Version 1.4.1 (Applera Corporation, Norwalk, CT, USA).

2.3. Sample preparation

Whole blood calibration, quality control and patient samples (100 μl) were treated with 100 μl of 0.3 mol/l zinc sulphate:methanol mixture (1:5, v/v) in the presence of the internal

Table 1
Compound dependent parameters of the HPLC–tandem mass spectrometry pesticide assay.

Pesticide [acquisition time]	Mass transition (*m*/*z*)	Dwell (ms)	Declustering potential (V)	Collision energy (eV)
Period 1 [0–2 min]				
Dimethoate (quantifier)	230.1 → 125.3	100	50	35
Dimethoate (qualifier)	230.1 → 143.1	100	50	35
Dimethoate-dimethyl-d_6 (IS[a])	235.9 → 131.0	100	50	35
Period 2 [2–5 min]				
Fenthion (quantifier)	279.1 → 169.1	75	20	30
Fenthion (qualifier)	279.1 → 104.9	75	20	30
Fenthion-dimethyl-d_6 (IS)	285.1 → 169.0	75	20	30
Diazinon (quantifier)	305.1 → 168.9	50	20	30
Diazinon (qualifier)	305.1 → 153.0	50	20	30
Diazinon-diethyl-d_{10} (IS)	315.2 → 170.1	50	20	30
Period 3 [5–8 min]				
Chlorpyrifos (quantifier)	350.0 → 197.9	100	20	30
Chlorpyrifos (qualifier)	351.9 → 200.0	100	20	30
Chlorpyrifos-diethyl-d_{10} (IS)	360.4 → 131.1	100	20	30

[a] Internal standard.

standards dimethoate-dimethyl-d_6 (2 ng/ml), fenthion-dimethyl-d_6 (10 ng/ml), diazinon-diethyl-d_{10} (2 ng/ml) and chlorpyrifos-diethyl-d_{10} (1 μg/ml). The mixture was vortex mixed for 2 min and centrifuged (5 min, 20,800 × *g*). A portion of the supernatant (20 μl) was injected into the HPLC–MS/MS system for analysis.

2.4. Assay validation studies

The selectivity of the method was assessed for potential endogenous interferences by analyzing whole blood samples (extracted without internal standard) from 20 subjects with no prior history of pesticide exposure. A peak or response at the respective retention times for all pesticides and their internal standards with a signal to noise ratio (S/N) of <5:1 was considered to be insignificant. Potential sample carry-over effects were studied by injecting an extract containing all pesticides (100 ng/ml) and internal standards followed by four blank extracts and observing any potential residual peaks at the retention times for each analyte. The process was repeated five times.

Linearity was evaluated by the analysis of whole blood calibration samples at concentrations of 0.5, 1.0, 5.0, 10, 25, 50 and 100 ng/ml ($n = 5$). A weighted linear regression model ($1/\chi^2$) was used throughout the study for construction of calibration curves. The inter-day accuracy and precision (expressed as the coefficient of variation, CV) of calibration samples were determined from the back-calculated results of the linearity study. The analytical performance of the method was further assessed based on the accuracy and precision of quality control samples at the lower limit of quantification of 0.5 ng/ml, within the linear range (2.5, 20, 75 ng/ml) and at the upper limit of quantification (100 ng/ml). Control samples were analyzed in replicates of 5 on 1 day (intra-day) and once on 5 days (inter-day). Accuracy was determined as the mean assayed result for the quality control samples ($n = 5$) expressed as a percentage of the weighed-in concentration. Precision was calculated as the standard deviation over the mean, expressed as a percentage. Dilution suitability for samples above the linear range (250 and 2500 ng/ml), were assessed based on 1 in 5 (50 μl:200 μl) and 1 in 50 (50 μl:2450 μl) dilutions with pesticide free blood. Intra-day accuracy and precision of diluted samples were determined by assaying quality control samples in replicates of 5 within 1 day.

The mean absolute recoveries of the analytes were determined by comparing the peak areas obtained from each pesticide (2.5, 20, 75 ng/ml) and their internal standards added to and extracted from whole blood samples of different subjects ($n = 5$) for each concentration, compared to the peak areas obtained from the analytes added post-extraction to their respective subject blank extracts. Post-extraction stability at 4 °C was determined by comparing results obtained at time 0 and 12 h for quality control samples at (2.5, 20, 75 ng/ml) analyzed in replicates of five. Inter-subject variability (as a measure of matrix effects) for all pesticides were determined by the measurement of the absolute recoveries using 5 of the 15 different subjects for each of three concentrations (2.5, 20 and 75 ng/ml)

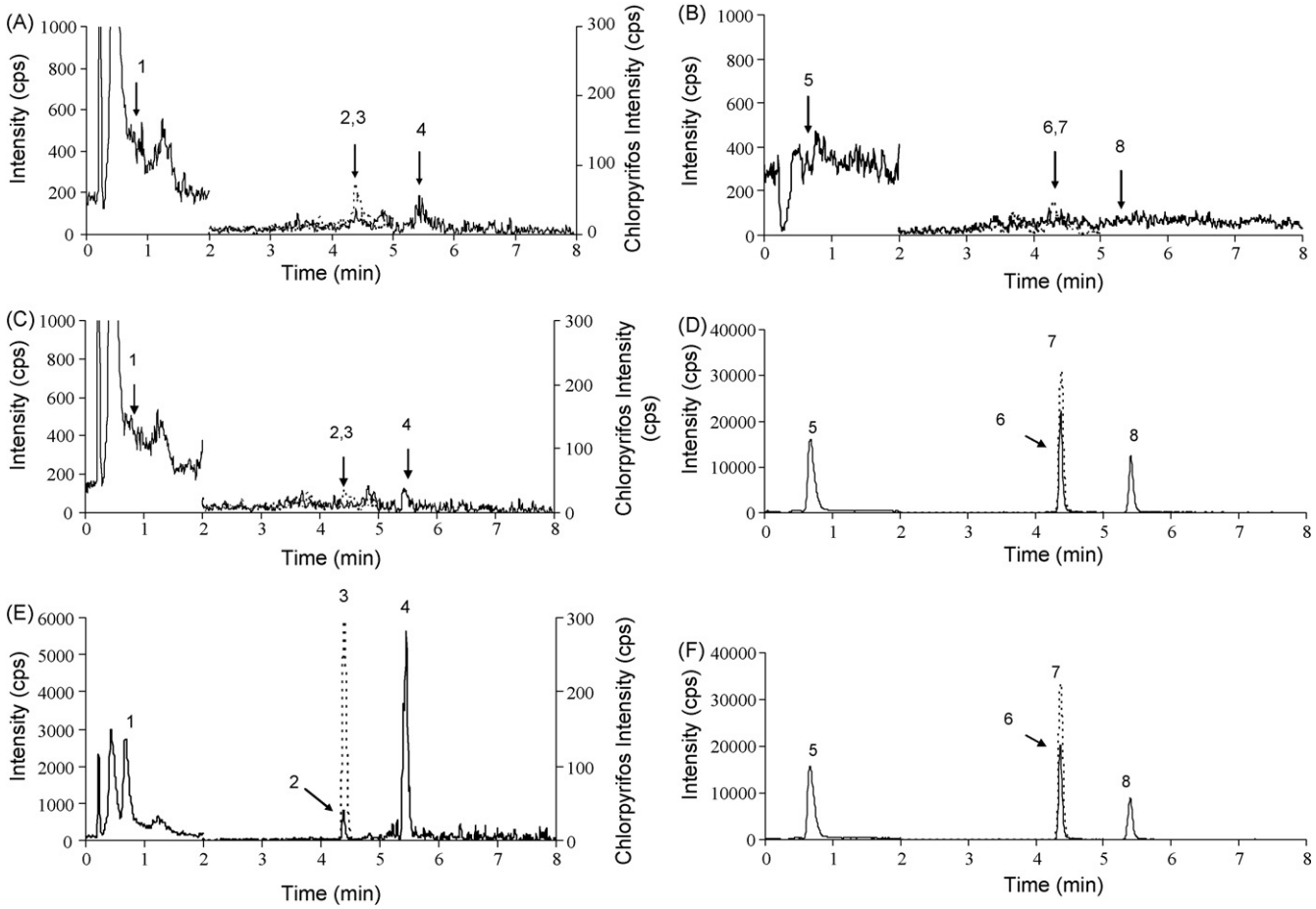

Fig. 1. Representative chromatograms of a blood extract from a blank (A) pesticide mass transitions trace and (B) internal standard mass transitions trace. Similarly, chromatograms from an extract of a blank blood sample with internal standard addition (C) pesticide mass transitions trace and (D) internal standard mass transitions trace. In addition, chromatograms from a blood extract at the lower limit of quantification (0.5 ng/ml) (E) pesticide mass transitions trace and (F) internal standard mass transitions trace. Pesticide peaks: 1, dimethoate; 2, fenthion; 3, diazinon (dotted line); 4, chlorpyrifos. Internal standard peaks: 5, dimethoate-dimethyl-d_6; 6, fenthion-dimethyl-d_6; 7, diazinon-diethyl-d_{10} (dotted line); 8, chlorpyrifos-diethyl-d_{10}.

and expressed as the coefficient of variation. As a confirmation measure, analytical specificity was determined based on the ratio between the peak area of the quantifier mass transition and the respective qualifier mass transition for each pesticide.

2.5. Application

The method was applied to identify and quantify the systemic OP exposure in a 28-year-old woman who was presented to a rural hospital in Sri Lanka within 2 h of acute intentional self-poisoning with diazinon. She developed signs of acute cholinergic toxicity and was treated with intravenous atropine. She recovered and was discharged home alive and well 4 days post-ingestion. Blood samples were collected at 2.25, 3, 6, 14, 36 and 58 h post-ingestion to determine the peak concentration and elimination half-life of diazinon. All samples were stored at −20 °C until the time of analysis.

3. Results and discussion

3.1. Sample preparation

A simple sample precipitation approach was undertaken where initial experiments involved blood pre-treatment with zinc sulphate (0.1 mol/l) and acetonitrile at various proportions in order to optimize extraction efficiency and minimize potential interference and signal suppression. Fenthion, diazinon and chlorpyrifos showed suitable extraction recoveries with no interferences, however dimethoate suffered from an interference at its expected retention time. As an alternative, a zinc sulphate (0.3 mol/l)/methanol mixture (1/5 parts) was used [25] which provided a suitable option for the extraction of all pesticides without compromising peak purity.

The simple one-step protein precipitation sample extraction procedure has labor and consumable cost savings over GC–mass spectrometry based methods that require liquid/liquid or solid phase sample extraction steps. This assay offers several benefits over other reported HPLC–MS/MS methods that also use protein precipitation for OP measurement [21,24], including the lack of complex column switching systems and the absence of a filtration step prior to injection. In addition, the analytical approach employed in this study in combination with the instrumentation used, has achieved lower limits of quantification compared to these methods [21,24].

3.2. Chromatography

The mobile phase was acidified to enhance protonation of the analytes. To achieve adequate column retention for dimethoate, the initial starting conditions comprised of 40% organics. The composition was subsequently changed to 80% organics to elute the other pesticides within a suitable analysis time. Therefore if the measurement of only fenthion, diazinon and chlorpyrifos were required, the chromatographic run time could be potentially shortened by 3.5 min per sample. This could be achieved by removing the initial 40% organic step and the final re-equilibration step and substituting the mobile phase with a composition of ∼70% organics in isocratic mode.

The chromatographic conditions used in this method achieved the following retention times for each pesticide and their respective internal standards; 0.7 min (dimethoate), 4.4 min (fenthion and

Table 2
Analytical performance of the HPLC–MS/MS method in dimethoate and fenthion whole blood control samples ($n = 5$).

	Dimethoate quality control concentration (ng/ml)				
	0.5	2.5	20	75	100
Accuracy[a] (%)					
Intra-day ($t = 0$ h)	98.6	99.5	102.0	102.3	96.7
Intra-day ($t = 12$ h)	–	99.5	100.4	100.6	–
Inter-day	99.4	101.1	100.6	101.0	98.7
Precision[b] (CV%)					
Intra-day ($t = 0$ h)	4.0	2.0	1.8	1.6	2.1
Intra-day ($t = 12$ h)	–	1.8	2.5	2.2	–
Inter-day	3.1	3.0	1.0	2.8	1.5
Absolute[c] recovery ± SD (%)	–	97 ± 2	100 ± 1	99 ± 3	–
Inter-subject[d] variability (CV%)	–	2.0	0.8	3.4	–
	Fenthion quality control concentration (ng/ml)				
	0.5	2.5	20	75	100
Accuracy[a] (%)					
Intra-day ($t = 0$ h)	102.5	103.2	101.2	102.7	103.6
Intra-day ($t = 12$ h)	–	101.7	100.8	95.1	–
Inter-day	98.4	105.1	99.8	96.6	104.8
Precision[b] (CV%)					
Intra-day ($t = 0$ h)	3.2	2.3	2.2	1.7	2.5
Intra-day ($t = 12$ h)	–	4.1	3.3	1.2	–
Inter-day	9.4	3.5	3.8	3.3	2.5
Absolute[c] recovery ± SD (%)	–	99 ± 4	97 ± 2	95 ± 2	–
Inter-subject[d] variability (CV%)	–	3.7	1.8	2.3	–

[a] Accuracy was determined as the mean measured concentration divided by the weighed-in concentration, expressed as a percentage. Intra-day accuracy determined in freshly prepared extract ($t = 0$ h) and stored extract in the autosampler at 4 °C ($t = 12$ h).

[b] Precision (coefficient of variation, CV) was calculated as the standard deviation divided by the mean, expressed as a percentage. Intra-day precision determined in freshly prepared extract ($t = 0$ h) and stored extract in the autosampler at 4 °C ($t = 12$ h).

[c] Absolute recovery (extraction efficiency) of the analytes was determined by comparing the independent peak areas obtained from each analyte added pre- and post-extraction.

[d] Inter-subject variability was expressed in terms of coefficient of variation (CV) in the absolute recoveries of different subjects.

diazinon) and 5.4 min (chlorpyrifos), with a total analysis time of 8 min. The additional 2.5 min after chlorpyrifos elution was required in order to achieve gradient re-equilibration before the next injection. Under the conditions employed, a higher concentration of chlorpyrifos-diethyl-d_{10} was required as its sensitivity was considerably less with respect to the other internal standards used. Representative chromatograms of an extract from a blank blood sample are shown in Fig. 1(A and B). Similarly, chromatograms of an extract of a blank blood sample with internal standard addition are shown in Fig. 1(C and D). The absence in the detection of OPs (Fig. 1C) in the presence of their respective labelled internal standards, confirms the purity of the internal standards used. In addition, chromatograms from a control sample prepared at the lower limit of quantification (0.5 ng/ml) are shown in Fig. 1(E and F).

3.3. Analytical specificity ratio (confirmation)

As has been recently recommended [26,27], at least two mass transitions for each analyte should be monitored where possible. Furthermore, the quality of the transitions chosen requires careful consideration, as non-specific losses such as H_2O or CO_2 can increase the risk of false positive or false negative confirmation results [17]. In particular, several approaches in assessing analytical specificity have been described [28]. In this study the monitoring of a secondary product ion of a parent ion with the same collision energy in the cases of dimethoate, fenthion and diazinon was employed. However an alternative approach was used for chlorpyrifos, where an isotopic parent ion and isotopic product ion at the same collision energy was used as the secondary mass transition. The alternative process for chlorpyrifos was preferred because both the precursor monoisotopic molecular ion (m/z 350.0) and the isotopic ion (m/z 351.9) had similar relative intensities. The major product ion from the isotopic molecular ion m/z 351.9 was m/z 200.0, which had a greater intensity than the minor monoisotopic product ions m/z 153.1 (600% higher) and m/z 125.0 (50% higher). Therefore the capability of measuring two mass transitions of similar intensities and assessing their confirmation ratios would improve the ability to detect potential interference from background noise or endogenous matrix components at low concentrations. Low intensities of qualifier ions at low concentrations (2.5 ng/ml) resulted in variable confirmation ratios for all OPs except for chlorpyrifos. Accordingly, the mean (±SD) area ratio of quantifier to qualifier mass transitions of control samples at 20 and 75 ng/ml for the following pesticides (n = 10, CV <3%) were 6.6 ± 0.19 (dimethoate), 1.8 ± 0.03 (fenthion) and 1.1 ± 0.03 (diazinon). Precise chlorpyrifos confirmation ratios could be determined at 2.5, 20 and 75 ng/ml with a specificity ratio of 1.0 ± 0.02 (n = 15, CV = 2%). These analytical specificity ratios were used for confirmation purposes.

3.4. Validation

No significant peaks or response (all S/N ratios were <5:1) were detected at the respective retention times for each pesticide or their respective internal standards in the screening of potential endogenous interferences (n = 20) or in the assessment of carry-over effects (n = 20). The assay was linear over the range 0.5–100 ng/ml (r^2 > 0.992, n = 24) for all pesticides. Accuracy and precision of all samples used in the construction of calibration curves were 93.8–105.0% with a CV <7%, respectively. The lower limit of quantification for each pesticide was determined as 0.5 ng/ml with an

Table 3
Analytical performance of the HPLC–MS/MS method in diazinon and chlorpyrifos whole blood control samples (n = 5).

	Diazinon quality control concentration (ng/ml)				
	0.5	2.5	20	75	100
Accuracy[a] (%)					
Intra-day (t = 0 h)	104.4	103.5	103.1	99.0	98.8
Intra-day (t = 12 h)	–	102.6	100.2	99.2	–
Inter-day	99.2	104.5	100.5	97.7	98.5
Precision[b] (CV%)					
Intra-day (t = 0 h)	3.8	1.8	1.9	1.6	1.9
Intra-day (t = 12 h)	–	3.9	0.8	2.4	–
Inter-day	7.5	2.5	2.1	1.4	4.3
Absolute[c] recovery ± SD (%)	–	97 ± 6	99 ± 1	98 ± 2	–
Inter-subject[d] variability (CV%)	–	5.8	1.2	1.8	–
	Chlorpyrifos quality control concentration (ng/ml)				
	0.5	2.5	20	75	100
Accuracy[a] (%)					
Intra-day (t = 0 h)	98.1	102.1	105.7	104.3	98.9
Intra-day (t = 12 h)	–	101.1	101.5	99.9	–
Inter-day	101.0	99.8	100.7	101.5	106.1
Precision[b] (CV%)					
Intra-day (t = 0 h)	3.6	5.5	1.4	1.8	5.6
Intra-day (t = 12 h)	–	10.8	9.4	9.8	–
Inter-day	7.2	6.4	4.9	5.2	8.0
Absolute[c] recovery ± SD (%)	–	97 ± 5	100 ± 1	99 ± 3	–
Inter-subject[d] variability (CV%)	–	5.5	0.9	3.0	–

[a] Accuracy was determined as the mean measured concentration divided by the weighed-in concentration, expressed as a percentage. Intra-day accuracy determined in freshly prepared extract (t = 0 h) and stored extract in the autosampler at 4 °C (t = 12 h).

[b] Precision (coefficient of variation, CV) was calculated as the standard deviation divided by the mean, expressed as a percentage. Intra-day precision determined in freshly prepared extract (t = 0 h) and stored extract in the autosampler at 4 °C (t = 12 h).

[c] Absolute recovery (extraction efficiency) of the analytes was determined by comparing the independent peak areas obtained from each analyte added pre- and post-extraction.

[d] Inter-subject variability was expressed in terms of coefficient of variation (CV) in the absolute recoveries of different subjects.

intra- and inter-day accuracy of 98.1–104.4% and precision CV <10%. However the true lower limit of quantification may not have been reached, as analytical performance was not tested at concentrations below the lowest calibrator (0.5 ng/ml). Freshly prepared quality control samples within the linear range (2.5, 20 and 75 ng/ml) had intra- and inter-day accuracy of 96.6–105.7% and precision CV <7%. The upper limit of quantification was deemed to be 100 ng/ml with intra- and inter-day accuracy of 96.7–106.1% and precision CV <9% (Tables 2 and 3).

Dilution suitability for samples outside the calibration range based on a 1 in 5 (250 ng/ml, $n = 5$) and a 1 in 50 dilution (2500 ng/ml, $n = 5$) gave acceptable intra-day accuracy (94.3–111.2%) and precision (CV <9%). This extended range would reasonably accommodate many instances of acute poisoning. However, further dilution validation may be required if higher concentrations were encountered, as has been cited in a case of fenthion poisoning, where a post mortem blood sample of 3800 ng/ml was recorded [20].

The analytical performance characteristics displayed by previously reported GC–MS/MS or HPLC–MS/MS methodologies for these pesticides in serum [21,22,24] in terms of lower limits of quantification (0.6–1250 ng/ml), accuracy (71–105%) and precision (CV <12%) compares satisfactorily with this current assay. Furthermore, based on the guidelines of Shah et al. [29] and current regulatory opinion [30] the assay displayed acceptable accuracy and precision for all quality control samples.

The collective mean (±SD) absolute recoveries for all concentrations studied ($n = 15$) were 98 ± 2% (dimethoate), 97 ± 3% (fenthion), 98 ± 3% (diazinon) and 99 ± 4% (chlorpyrifos). Similarly, the mean absolute recoveries for their respective internal standards were >96%. The absolute recoveries obtained for these pesticides in this work were similar to other studies which used protein precipitation with typical recoveries of 90–110% [21,24]. Therefore, the extraction process of whole blood samples treated with a solution containing one part zinc sulphate (0.3 mol/l) to five parts methanol was an effective precipitation and extraction reagent.

The post preparative stability of the pesticides was investigated for the purpose of determining the appropriate storage requirements for sample extracts during an analytical run. Initial investigations at room temperature, exposure to light and the use of plastic vial inserts, revealed a noticeable decline in absolute peak area intensity for chlorpyrifos and its internal standard of up to 30%, while the other pesticides appeared to be unaffected. Therefore, sample extract storage at 4 °C using amber vials with glass inserts was assessed. As shown in Tables 2 and 3, the results from sample extracts ($n = 5$) from quality control samples at concentrations of 2.5, 20 and 75 ng/ml, injected after storage in an autosampler for a 12-h period, exhibited an accuracy and precision of 95.1–102.6% and CV <11%, respectively. These performance measures compare favorably with the validation data of freshly prepared extracts. This indicates that these pesticides are stable in their post preparative form for at least 12 h at 4 °C in amber vials.

As suggested by Matuszewski et al. [31] investigations into inter-subject variability should be performed to assess the influence of matrix differences on assay performance and is strongly recommended as an important component to HPLC–MS/MS validation [32,33]. The results of this study show precision CV of <6% between different subjects for each pesticide ($n = 15$), which compares favorably with quality control samples made from a single blood pool with a precision CV of <10% (Tables 2 and 3). These similar results in precision confirm that the assay was not significantly influenced by biological variations in blood from different subjects. These data are consistent with the lack of matrix effects adversely influencing results, largely due to the use of stable isotope internal standards, which are expected to compensate (or correct) for variations in ionization efficiency.

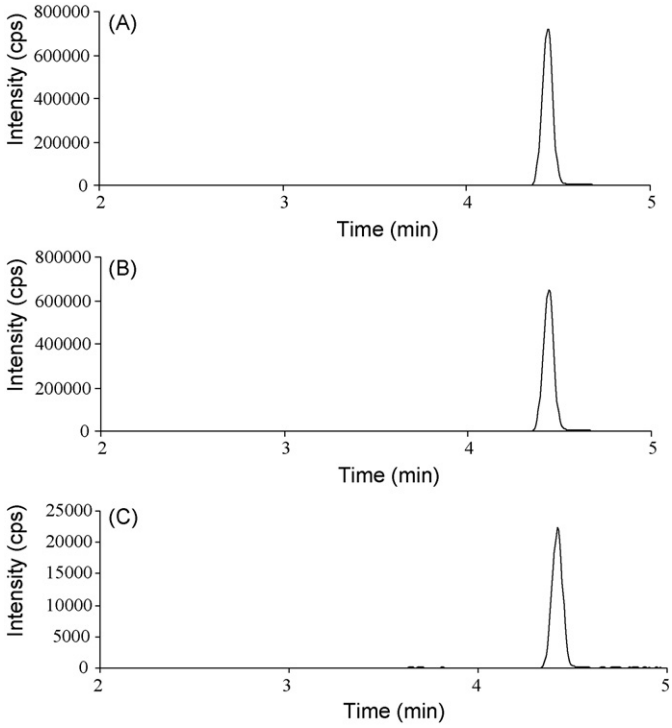

Fig. 2. Chromatograms of a blood extract from a 28-year-old female subject 58 h after acute diazinon poisoning (91.6 ng/ml). (A) Quantification mass transition trace (m/z 305.1 → 168.9), (B) qualification mass transition trace (m/z 305.1 → 153.0) and (C) internal standard mass transition trace (m/z 315.2 → 170.1).

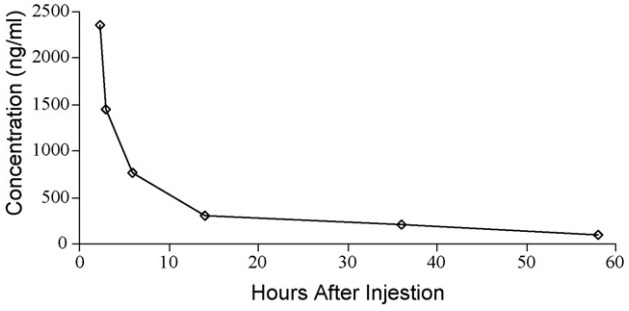

Fig. 3. Diazinon blood concentration–time profile of a 28-year-old female subject after acute poisoning.

3.5. Application

Diazinon poisoning was confirmed in a chromatogram of the blood extract provided by a woman presenting with acute self-poisoning, with a concentration of 91.6 ng/ml (confirmation ratio of 1.10, Fig. 2). The concentration–time profile in Fig. 3 (91.6–2360 ng/ml) suggests a biphasic elimination profile with an initial half-life of 1.6 h and a terminal half-life of 26 h. The analytical specificity ratios in these samples ranged from 1.10 to 1.13 ($n = 6$). The similar specificity ratios of diazinon control samples (1.1) compared with this patient provides additional confirmation of diazinon poisoning.

4. Conclusion

We have described a HPLC–MS/MS assay for the simultaneous quantification and confirmation of dimethoate, fenthion, diazinon and chlorpyrifos in human blood, with a simplified extraction

procedure that offers improvements over previous methods [21,24]. This assay displays suitable analytical performance characteristics over a concentration range that could be applied to cases of acute poisoning.

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