Analysis of Fenthion in Postmortem Samples by HPLC with Diode-Array Detection and GC–MS Using Solid-Phase Extraction

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Abstract

Fenthion (O,O-dimethyl-O-[3-methyl-4-(methylthio)-phenyl]-thiophosphate) is an organophosphate insecticide. A specific method to quantitate fenthion in postmortem matrices with solid-phase extraction combined with high-performance liquid chromatography–diode-array detection (HPLC-DAD) and gas chromatography–mass spectrometry (GC–MS) is presented. Fenitrothion (O,O-dimethyl-O-[3-methyl-4-nitrophenyl]-thiophosphate) is selected as the internal standard. For sample cleanup, a simple but selective solid-phase extraction is chosen after comparison with traditional liquid-liquid extraction procedures. Homogenized and appropriately diluted aqueous samples are applied, and the analytes are desorbed with 5 mL of dichloromethane. Aliquots of the extract are used for HPLC-DAD and GC-MS analysis. Liquid and GC conditions are as follows: gradient elution with a mixture of methanol and water (10:90 to 90:10, v/v) containing 0.0125M NaOH on an Aluspher RP-Select B column monitoring at 250 nm, and temperature programming from 60 to 300°C on a dimethylpolysiloxane column in the SCAN mode, respectively. This method is applied to a suicidal case involving unsuspected acute intoxication with fenthion (concentration in blood, 3.8 µg/mL).

Numerous cases of intoxication with organophosphate insecticides have occurred because of their widespread use and accessibility (3). Nevertheless, only one case of death resulting from an overdose by oral ingestion of fenthion has been investigated analytically to date (4). Exposure to organophosphate insecticides can be either accidental (following occupational exposure) or intentional (with either a suicidal or a homicidal purpose). Acute intoxications following oral ingestion typically result in symptoms such as nausea, vomiting, diarrhea, convulsions, hypotension, coma, and central respiratory paralysis (1,2). This paper describes the development of an analytical method for fenthion. Solid-phase extraction (SPE) was used for sample cleanup, and identification and quantitation were performed using specific high-performance liquid chromatography–diode-array detection (HPLC-DAD) and gas chromatography–mass spectrometry (GC–MS) methods. These methods were applied to determine the tissue distribution of fenthion in a forensic case that was recently presented in our laboratory.

Experimental

Solvents and reagents
Fenthion, fenitrothion, and fensulfothion were obtained from the Pestanal® collection of Riedel-deHaen distributed by CRB (Brussels, Belgium). The solid-phase columns were 200-mg Bond Elut C-18 columns from Varian (Sunnyvale, CA). All solvents were of analytical-grade purity. Methanol, n-hexane, and ethylacetate were obtained from Merck (Darmstadt, Germany); HPLC-grade water was from Romil (Loughborough, U.K.); and dichloromethane was from Sigma-Aldrich (Steinheim, Germany). Stock solutions containing fenthion and fenitrothion at concentrations of 1.0 and 2.2 mg/mL, respectively, were prepared in methanol. Diazomethane in diethyl ether was synthesized in our laboratory (5).

Fenthion determination

Internal standard. In order to allow for reliable quantitative data, an appropriate internal standard was selected. Two commercially
available structural analogues of fenthion were evaluated for this purpose. These two compounds were fenitrothion, or O,O-di-
dimethyl-O-(3-methyl-4-nitrophenyl)-thiophosphate, and fensul-
ofothion, or O,O-diethyl-O-(4-methylsulfonylphenyl)-thiophosphate (Figure 1). After injection on both the HPLC–DAD and
gc–MS systems, fenitrothion showed superior LC and GC
behavior compared with fensulfothion, which chromatographed
twice as asymmetric peaks. Fenitrothion was therefore chosen as
the internal standard. Baseline separation of fenthion and fen-
itrothion was easily obtained with the conditions described for the
HPLC–DAD and GC–MS systems.

Extraction and derivatization. Initially, the use of a liquid–
liquid extraction procedure was evaluated. Experiments were
performed on blank whole-blood samples spiked with fenthion.
Results with different mixtures of n-hexane and ethylacetate
(70:30, 50:50, and 30:70, v/v) at pH values of 4.0, 6.0, and 9.5 were
all unsatisfactory because of the low and irreproducible recov-
eries of fenthion. Using ether as the extraction solvent after acid-
ification with 2M hydrochloric acid until pH 2.0 (6), emulsions
were obtained as expected for blood. This problem was largely
overcome by adding sodium chloride before extraction on a
rotamixing device. However, fenthion extraction recoveries were
again low and not reproducible.

An SPE protocol described by Psathaki et al. (7) for trace
enrichment of various pesticides in water samples was evaluated
for the cleanup of fenthion in forensic whole blood. Except for
the immediate addition of the internal standard before extrac-
tion, only minor modifications were performed. Briefly, solid-
phase cartridges were flushed with 5 mL of dichloromethane and
subsequently conditioned with 5 mL of methanol followed by 3
mL of HPLC-grade water. One-milliliter spiked samples
(adjusted to pH 7 when necessary) were diluted to a total volume
of 5 mL with HPLC-grade water, ultrasonicated, and applied
slowly on the Bond Elut C-18 cartridges. Cartridges were then
dried under maximum vacuum for 5 min, and the analytes were
desorbed with one 5-mL portion of dichloromethane. The elu-
tion solvent was evaporated under nitrogen, and the extraction
residue was redissolved in 150 μL of a methanol/water mixture
(50:50, v/v). Half of the extract was injected in the HPLC–DAD
system, and the remaining volume was used for GC–MS analysis.
It was dried under a flow of nitrogen, and 50 μL of an ethereal
solution of diazomethane was added. The reaction took place at
room temperature over 5 min. After a second evaporation step
under nitrogen, the residue was redissolved in 50 μL of ethylac-
etate, and 1 μL was injected into the GC–MS system. For urine
and other matrices, a similar procedure was followed. Total
sample volume was always adjusted to 5 mL. Tissue samples were
homogenized with an Ultra-Turrax mixer after dilution (1:1,
w/w) with HPLC-grade water. As with whole blood, 2-g aliquots
of tissue homogenates (stomach contents, liver, and kidney)
were submitted to ultrasonication for 15 min before centrifuga-
tion at 2500 rpm for 10 min. SPE was performed on the obtained
supernatant.

Chromatographic conditions: HPLC–DAD system. The gra-
dient HPLC system consisted of a model 168 DAD and a type
210A manual injector fitted with a 50-μL sample loop from
Beckman (Analis, Gent, Belgium). The photodiode-array
detector was operated in a 4-nm band-pass mode, monitoring
light from 225 to 350 nm. The usual display wavelength of 230
nm was changed to 250 nm. The chromatographic separation
was achieved on an Aluspher® RP-select B column (12.5 cm x
4.0-mm i.d., 5-μm particle size) protected by a Lichrocart® Aluspher 60 RP-select B (4 mm x 4-mm i.d., 30-μm particle
size) guard column. Both the analytical and guard columns
were from Merck (Darmstadt, Germany) and
were fitted manually by the Manu-Fix device
(Merck). The mobile phase was a mixture of
0.0125M NaOH in methanol (solvent A) and
0.0125M NaOH in HPLC-grade water (solvent
B). The following gradient conditions were
used: isocratic at 10% A for 5 min, then a linear
gradient to 90% A over 15 min, followed by a
hold at 90% A over 5 min. The total run time
was 25 min at a flow rate of 1.0 mL/min. At the
completion of the run, the pump was pro-
grammed to regain the initial conditions over
1 min. A 5-min reconditioning time was allowed
before the next injection.

Chromatographic conditions: GC–MS system.
The GC–MS system consisted of a series 3400
Varian GC (Sunnyvale, CA) in combination with a
Finnigan Mat (San José, CA) Magnum mass
selective ion-trap detector. An SGE (Achrom,
Zulte, Belgium) BPX5 capillary column (25 m x
0.22-mm i.d., 0.25-μm film thickness) was
installed in the GC. The injector and the trans-
fer line were held at 270 and 280°C, respec-
tively. The carrier gas was helium at a flow rate
of 0.8 mL/min. The initial oven temperature of
60°C was programmed to 120°C at 30°C/min,
increased to 220°C at 10°C/min, then programmed to 290°C at 5°C/min, and finally increased to 300°C where it was held for 3 min. One-microliter samples were injected in the splitless mode. Electron impact ionization and the SCAN mode were used in combination with a delay time of 200 s and an acquire time of 30 min.

**Quantitative analysis.** Separate calibration graphs were prepared in blank matrices for all of the different matrix types. Each sample was spiked with 50 µL of the 0.2-µg/µL internal standard solution (fenitrothion). Liver samples were spiked with 50 µL of the 0.4-µg/µL solution. The added fenthion levels for each calibration graph were 0, 2.0, 4.0, 6.0, 8.0, and 10.0 µg/mL for blood and stomach contents and 0, 4.0, 6.0, 8.0, 12.0, 16.0, and 20.0 µg/g for liver homogenate. A ratio of approximately 1 was always obtained in the middle of each calibration graph. Forensic case samples were analyzed twice. First, 1-mL or 2-g homogenous aliquots of all available postmortem matrices (blood, urine, stomach contents, liver, and kidney) were spiked with the internal standard solution and extracted. Following these orientating preliminary results, a second extraction was performed on appropriately diluted sample specimens.

**Results and Discussion**

For the accurate quantitation of fenthion in postmortem matrices, a suitable internal standard and a specific extraction in combination with optimized chromatographic conditions were required. First, the internal standard selection was performed as described in Experimental. Fenitrothion, a structural analogue of fenthion (Figure 1), chromatographed baseline separated from fenthion in both chromatographic systems (Figures 2 and 3). The extraction step was then optimized because the routine liquid–liquid extraction method used for initial sample screening had a recovery of less than 20% for fenthion and the internal standard. Moreover, a peak interfering with fenthion was present in the HPLC chromatograms of blood extracts, indicating a nonsel ective sample cleanup. After comparison with modified and alternative liquid–liquid extraction procedures, the solid-phase procedure as described in Experimental was selected for sample extraction. These simple solid-phase conditions resulted in good recoveries of both fenthion and the internal standard from spiked whole blood (Table I). Recoveries from water and liver homogenate were higher and lower, respectively, than blood. Recoveries were not concentration dependent. Calibration curves prepared in whole blood, water, and liver homogenate were linear in the selected range with good correlation coefficients (r = 0.999, 0.999, and 0.996, respectively), and the following equations were obtained using linear regression analysis: \( y = 0.209x + 0.038 \), \( y = 0.212x + 0.072 \), and \( y = 0.088x - 0.032 \), respectively.

As described in Experimental, the extract of each sample was used for both HPLC–DAD and GC–MS analyses. The solid-phase elution fraction was evaporated and redissolved in 150 µL of a methanol/water mixture, and an aliquot was injected directly onto the HPLC system. The remaining portion was evaporated, reacted with ethereal diazomethane, and redissolved in ethyl acetate after a second evaporation step. The derivatization step was included only to reduce peak tailing and enhance volatility of endogenous matrix constituents by methylation of the polar functional groups. The analytes of interest, fenthion and fenitrothion, remained underivatized because they did not react with diazomethane.

The chromatographic conditions described in Experimental were used for the LC and GC systems. These conditions were identical to the general

![Figure 2. HPLC–DAD chromatogram of the forensic liver sample extract monitored at 250 nm. The UV spectrum of fenthion is shown in the inset. Level of fenthion: 203 µg/g. Peak identification: 1, internal standard and 2, fenthion.](image)

![Figure 3. GC–MS chromatogram (TIC) of the forensic blood sample extract after derivatization. The mass spectrum of fenthion is shown in the inset. Level of fenthion: 3.8 µg/mL. Peak identification: 1, internal standard and 2, fenthion.](image)
screening conditions. However, for the HPLC–DAD system, one important modification was applied: the monitoring wavelength was changed from 230 to 250 nm. The HPLC chromatograms displayed at 250 nm showed a dramatic increase both in sensitivity ($\lambda_{max}$ of fenthion = 251 nm) and in selectivity when compared with the initial chromatograms monitored at 230 nm. The interfering peak that was observed in whole blood samples also disappeared when the wavelength was switched from 230 to 250 nm.

The described procedure was applied to real forensic samples from a suicidal case involving nonsuspected fenthion poisoning. The victim was a 66-year-old woman found dead in her bedroom. In accordance with the laboratory’s operating procedures, a comprehensive toxicological screening, including homogenous enzyme multiplied immunoassay and radioimmunoassay for a large number of drugs or classes of drugs and headspace analysis of volatiles and the Fujiwara–Ross reaction, was first performed on the blood and urine obtained at autopsy (8).

Following screening with thin-layer chromatography (TLC), no spots were detected in the extracts of blood and urine. Following screening with HPLC–DAD, a large peak with a retention time of 18.3 min and a typical UV spectrum was observed in the extracts of both blood and stomach contents. The identity of the compound with this UV spectrum was disclosed as fenthion by a library search. The peaks in the HPLC chromatograms of blood and stomach contents were also observed in the corresponding GC–MS chromatograms. These peaks had a retention time of 12.5 min and were identified by a mass spectral library search as fenthion.

Furthermore, the peaks in the HPLC and GC chromatograms coeluted and displayed identical UV and mass spectra as a fenthion standard, respectively (Figures 2 and 3). The UV spectrum of fenthion was characterized by an absorption maximum at 251 nm. The mass spectrum of fenthion showed limited fragmenta-

tion, the base peak being the molecular ion with a mass-to-charge ratio of 278 and the most prominent fragment ion having a mass-to-charge ratio of 245. Moreover, the cholinesterase activity was measured in blood, and a value of 202 U/L was found. This value indicated a strong cholinesterase inhibition (reference range: 3700–13,200 U/L) (9) and indirectly confirmed the presence of a cholinesterase inhibitor in blood. The autopsy observations also supported an acute cholinergic crisis due to overstimulation of the parasympathic autonomic nervous system (with symptoms like diarrhea and broncho-constriction). Finally, a semiquantitative TLC procedure described for the detection of various pesticides (10) was performed. This specific TLC procedure was necessary because fenthion could not be demonstrated in the forensic samples using the general TLC-screening conditions. Briefly, 2.5 mL of blood and urine and 10 mL of appropriately diluted stomach contents were extracted with n-hexane, spotted onto a silica-gel plate and developed with a mixture of benzene/methanol (9:1, v/v) as the mobile phase. Spots were then visualized by spraying with a 0.2% PdCl$_2$ solution. Spots with the same R$_f$ value (0.9) and color as those of a fenthion standard were observed in blood and stomach contents. No spot was detected in urine. The estimated fenthion concentration in blood was 2 to 3 µg/mL.

Following these preliminary identification experiments, fenthion was quantitated in all available postmortem samples. Results of fenthion concentrations have been summarized in Table II. In stomach contents, a fenthion concentration of 85 mg/mL (total amount: 17 g) was demonstrated, clearly indicating oral ingestion of the insecticide solution. This was confirmed by the presence of fluid with a strong organic solvent smell and a remarked blue color in the stomach contents, esophagus, mouth, and nostrils, which were observed at autopsy. The fenthion concentration in blood was 3.8 µg/mL, and a concentration of 203 µg/g was found in liver. Fenthion was present in urine and kidney, but the levels were very low and could not be quantitated. Quantitation limits (signal-to-noise ratio of 5:1) of fenthion in blood were 0.25 and 0.1 µg/mL with HPLC–DAD and GC–MS, respectively. When compared with the concentrations reported in the other fenthion case (4), a similar blood concentration was found (4.8 µg/mL), but the liver and kidney concentrations were different, 16.8 and 23.1 µg/g, respectively, from the present case. Fatal blood concentrations of organophosphates similar to fenthion have been reported (11,12). Fatal parathion blood levels were comparable with those of fenthion and varied from 0.5 to 34 µg/mL (mean of 9.0 µg/mL, n = 19), whereas malathion blood levels were much higher and varied from 175 to 517 µg/mL (mean of 281 µg/mL, n = 6).

Interestingly, small peaks with a UV spectrum identical to fenthion were observed in blood, urine, and kidney. These peaks presumably corresponded to a more polar fenthion metabolite formed by enzymatic biotransformation (1,2). Using GC–MS in the scan mode, more polar fenthion degradation products were indeed demonstrated in the three matrices mentioned here. Following a library search, minor peaks were tentatively identified as a fenthion-O-analogue (i.e., fenthion oxon) with a molecular ion with a mass-to-charge ratio of 262 (13). Moreover, more important peaks were also observed in the urine and kidney extracts and tentatively identified as 3-methyl-4-(methylthio)-phenol (Figure 1).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fenthion (µg/mL)</th>
<th>Fentrothion (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Stomach contents (total amount in g)</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Liver (µg/g)</td>
<td>203</td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>B.Q.L.*</td>
<td></td>
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<tr>
<td>Kidney</td>
<td>B.Q.L.</td>
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<tr>
<td><strong>Table I. Mean Recoveries (%) and Coefficient of Variation (%, in parentheses) of Fenthion and of Fenitrothion from Spiked Samples using Bond Elut C-18 Cartridges</strong></td>
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<th>Fentrothion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>71 (12.5)*</td>
<td>78 (5.5)*</td>
</tr>
<tr>
<td>Water</td>
<td>82 (4.8)*</td>
<td>87 (7.0)*</td>
</tr>
<tr>
<td>Liver</td>
<td>46 (8.0)*</td>
<td>62 (4.4)*</td>
</tr>
</tbody>
</table>

* n = 5.

* n = 6.

* n = 7.

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<tr>
<td><strong>Table II. Concentrations of Fenthion in Postmortem Specimens of an Acute Intoxication Case</strong></td>
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</table>

*Below the quantitation limit.
Conclusion

A method was developed for the analysis of fenthion in different biological matrices using SPE in combination with HPLC-DAD and GC–MS. It can be concluded from the experience with forensic samples in our laboratory that this method can be successfully applied for specific case work.

Acknowledgments

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References


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