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A fatal forensic intoxication with fenarimol: analysis by HPLC/DAD/MSD

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Abstract

Fenarimol (*Rubigan*®) is a pyrimidine ergosterol biosynthesis inhibitor used as a systemic fungicide. The authors present a fatal fenarimol intoxication case analysed in the Forensic Toxicology Service of the National Institute of Legal Medicine. The results were used to compare two different HPLC techniques, regarding selectivity and sensitivity: an HPLC system with a diode array detector (DAD) and an HPLC system with a DAD and a mass spectrometry detector (MSD) with an electrospray interface. All biological samples were submitted to a solid-phase extraction procedure. The detection and quantification limits of fenarimol, linearity, precision and accuracy were evaluated. The fenarimol concentration levels determined were of 89.0 mg/ml in gastric contents, 1.9 mg/g in liver and 0.4 mg/g in kidney. Blood was not available at autopsy. No published data related to fenarimol self-poisoning were found, so it was not possible to interpret the results obtained by comparison with toxic/lethal levels.

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1. Introduction

Fenarimol (2,4′-dicloro-α-(pyrimidin-5-yl) benzhydryl alcohol) [1], commercially denominated by *Rubigan*®, is an ergosterol biosynthesis inhibitor. It is used in agriculture, in doses of 120 g/l, as a systemic fungicide [2]. The compound is photochemically unstable, and is readily broken down by sunlight [1,3–5]. Fenarimol has shown reproductive, teratogenic, and oncogenic effects in experimental animals. It inhibits testosterone aromatase activity, which could result in an irreversible infertility in male rats [6–8]. Accidental human exposition could lead to, among others, eye irritation with corneal injury, skin irritation, nauseas, vomits and diarrhoea [9]. The lethal dose for an individual of 70 kg is estimated to be 28–500 g [10]. However, no reports on fatal cases have been published so far.

This work aimed at designing and validating in terms of linearity and recovery a method for the determination of fenarimol concentrations in liver, kidney and gastric content samples by HPLC/DAD/MSD.

2. Case report

A 46-year-old policeman was found, face down, in water surface inside an agricultural barrage. Close to the victim was found a pack of *Bladan*®, a common commercial name of parathion, an organophosphorus pesticide. The victim was under psychiatric treatment for depression. The autopsy report suggested intoxication with organophosphorus pesticides due to the presence of a strange scent in vomit and gastric content. The postmortem findings also indicated generalized visceral congestion and sero-mucous sanguine suffusion. No signs of violence were observed. Drowning was suggested as another possible cause of death. The autopsy was performed 3 days after the probable date of death. Biological samples received

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for toxicological analysis were stomach and its content, liver and kidney. The pack of *Bladan*® was also submitted to analysis.

3. Material and methods

3.1. Chemicals

All solvents were analytical or HPLC grade and were purchased from E. Merck (Darmstadt, Germany). Water was purified by a Milli-Q system obtained from Millipore (Molsheim, France). The fungicides fenarimol and ethirimol (used as internal standard) were supplied by Riedel-deHaën (Seelze, Germany). Each standard compound was dissolved in acetonitrile to perform a 1 mg/ml solution and stored at 4 °C. The solid-phase extraction columns were *Bond Elut Certify*® *LRC* obtained from Varian (Harbor City, USA), and were used on a solid-phase system *Vac Elut EPS 24*, Varian. The K_2CO_3 buffer (pH 9.5) was prepared by dissolving 13.8 g of K_2CO_3 into a 100 ml volumetric flask and bringing to volume with deionised water. The buffer was stored refrigerated (4 °C). Acetic acid solution (0.1 M) was prepared by adding 57.2 µl of glacial acetic acid ($d = 1.05$ g/l, $m = 60.05$ g) into a 100 ml volumetric flask and bringing to volume with deionised water. Control and calibration samples were prepared by spiking drug-free blood, liver and kidney postmortem samples with standard solutions.

3.2. Instrumentation

The chromatographic system used was a Waters 2690 Alliance System under software Millennium[32] v. 4.0 (Milford, USA) and a Symmetry® C_{18} (2.1 × 150 mm i.d., 3.5 µm). The mobile phase consisted of a mixture of acetonitrile and water (60:40, v/v) at a flow rate of 0.250 ml/min. The separation was performed at 30 °C. The injection volume was 2.5 µl.

Detection employed a Waters 996 photodiode array detector (DAD) operated in the 210–400 nm wavelength passband with a 1.2 nm resolution. The UV absorbance was measured at 225 nm.

Mass spectrometry detection (MSD) was carried out by a Waters ZQ 2000 mass spectrometer piloted using Millennium[32] v. 4.0 software and operated in the positive ionization mode in the *m/0* 180–380 mass range, with a scan time of 1 s and an interscan delay of 0.2 s. The mass spectra were represented in the centroid mode. The main other instrumental settings were: capillary voltage 3.5 kV; cone voltage 25 V; extractor 6 V; ion energy 0.1; source temperature 80 °C; desolvation temperature 110 °C; cone gas (N_2) flowrate 50 l/h and desolvation gas (N_2) flowrate 250 l/h.

3.3. Sample preparation

Blood, liver and kidney samples (1 ml or 1 g) and gastric content (0.5 ml) were homogenized in deionised water (4 ml). One millilitre of a K_2CO_3 buffer (pH 9.5) and 20 µl of ethirimol, internal standard, (1 mg/ml) were added to each sample and centrifuged at 2000 g for 15 min. Supernatants from these specimens were extracted by solid-phase extraction using *Bond Elut Certify*® *LRC* columns, previously conditioned with 5 ml methanol, 3 ml deionised water and 1 ml of 0.1 M acetic acid. After a 5 min drying step under vacuum, 2 ml *n*-hexane were applied to the column. Samples were deposited on the columns which were subsequently washed with 1 ml deionised water. After drying under vacuum for 10 min, elution was carried out with 3 ml of *n*-hexane/ethyl acetate (1:1, v/v). The eluate was collected into a conical glass tube and evaporated to dryness under a nitrogen stream (40 °C). The residue was reconstituted in

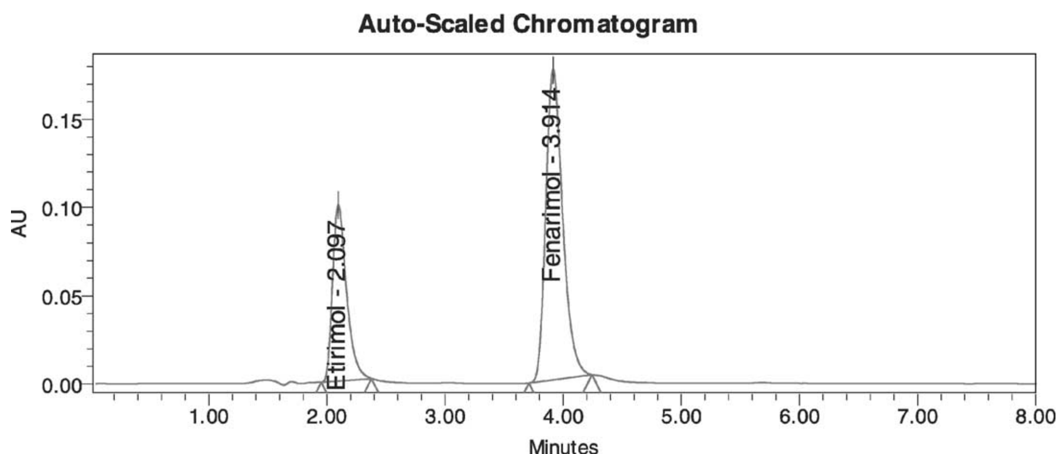

Fig. 1. Chromatogram obtained from a *postmortem* liver specimen spiked with fenarimol at 50 µg/g and ethirimol at 20 µg/g followed by DAD detection, at 225 nm.

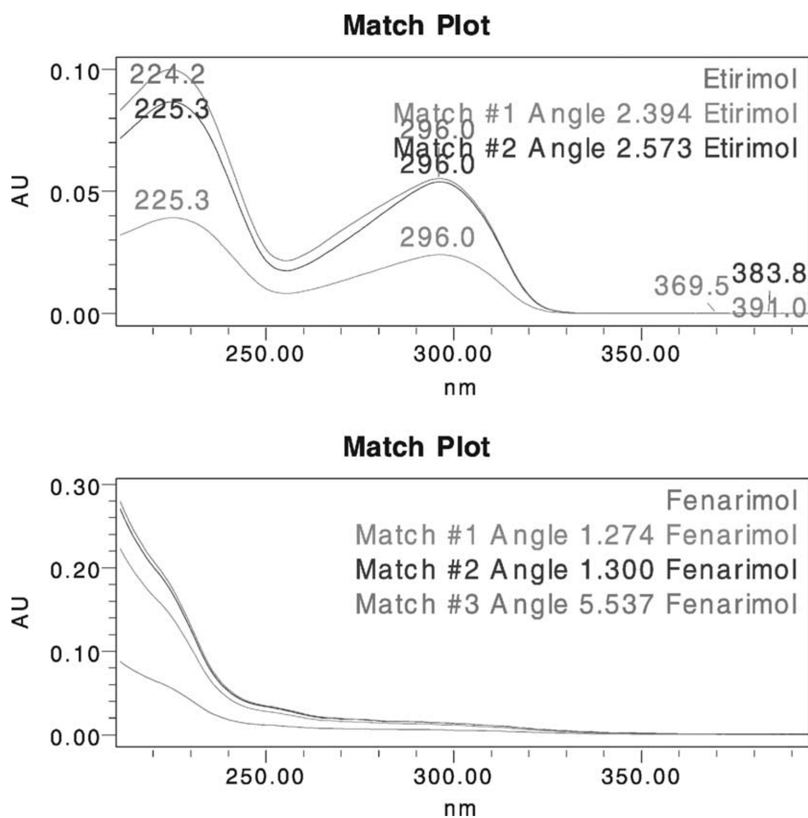

Fig. 2. UV spectral comparison of extracted fenarimol and ethirimol with a library obtain from a methanolic standard.

Table 1
Within-day precision and accuracy of fenarimol determination in blood, liver and kidney postmortem samples ($n = 5$)

Spiked concentration (μg/ml or μg/g)	Measured concentration (mean ± S.D.) (μg/ml or μg/g)	Precision (%CV)	Accuracy (%)
Blood			
5	4.83 ± 0.271	5.6	96.60
10	9.91 ± 0.158	1.6	99.10
20	19.23 ± 0.169	0.9	96.15
100	98.65 ± 1.127	1.1	98.65
250	247.90 ± 1.925	0.8	99.16
Liver			
5	4.72 ± 0.195	4.1	94.40
10	9.50 ± 0.154	1.6	95.00
20	18.52 ± 0.527	2.8	92.60
100	96.77 ± 1.840	1.9	96.77
250	240.45 ± 4.418	1.8	96.18
Kidney			
5	3.78 ± 0.173	4.6	75.60
10	8.20 ± 0.176	2.1	82.00
20	17.34 ± 0.577	3.3	86.70
100	82.20 ± 1.155	1.4	82.20
250	242.29 ± 10.702	4.4	96.92

1 ml of methanol, and an aliquot (2.5 μl) was injected in the HPLC system.

4. Results and discussion

Since intoxication by an organophosphorus pesticide was suspected and the package of *Bladan*® was found on the scene, an initial screen test by TLC was done. The pesticide parathion presence was confirmed on the pack found near the victim but not in postmortem samples.

Chromatographic conditions were optimised to provide both short retention times and an adequate peak shape in order to allow the quantification of fenarimol. Ethirimol, a fungicide not used in Portugal, was selected as internal standard. Fig. 1 shows a HPLC chromatogram obtained from a postmortem liver specimen spiked with fenarimol (RT = 3.914 min) at 50 μg/g and ethirimol (RT = 2.097 min) at 20 μg/g, followed by DAD detection at 225 nm. The UV spectrum of fenarimol and ethirimol is compared to a user-built library containing a standard spectrum of the same compound (Fig. 2).

Calibration curves of fenarimol in blood, liver and kidney samples were linear over a concentration range of 5–250 μg/ml, with correlation coefficients ≥0.9924 (six calibration points in triplicates). Linearity was also observed in the standard curves with a concentration range of 0.06 to 5 μg/ml (CV < 12). Standard curves were constructed by plotting a 1 per year weighted least-squares linear regression of fenarimol to the internal standard peak-area ratios versus the spiked concentration of fenarimol.

The validation results are summarised in Table 1. Within-day precision was evaluated by replicate analysis ($n = 5$) of spiked blood, liver and kidney samples, at each of five concentrations. The concentrations used were based on the high concentrations of fenarimol found in postmortem

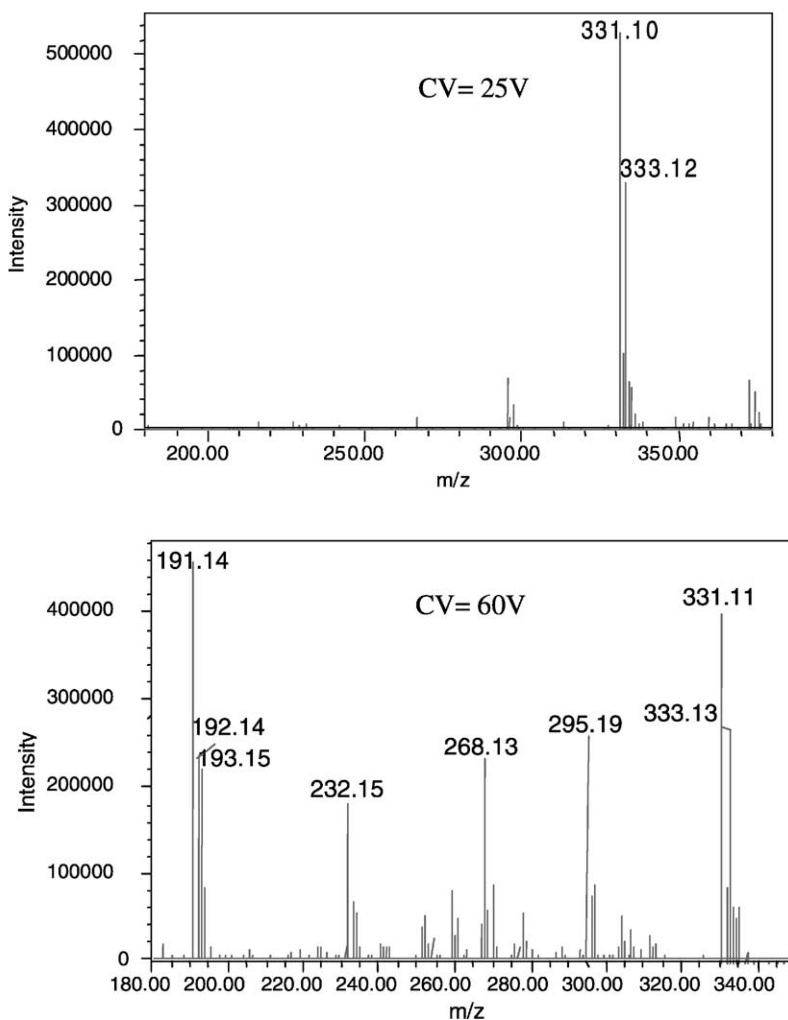

Fig. 3. Positive ES mass spectrum of fenarimol: cone voltage of 25 and 60 V.

samples. In blood samples, the within-day coefficients of variation (%CV) ranged between 0.8 and 5.6%, and accuracy between 96.5 and 99.2%. The within-day %CV for the liver samples ranged from 1.6 to 4.1 and for the kidney samples the %CV values were less than 4.6%. Accuracy for liver and kidney samples was in range 92.6–96.8% and 75.5–96.9%, respectively.

The limits of detection (LOD) were evaluated by analysing drug-free samples spiked with fenarimol at decreasing concentrations, until a response equivalent to three times the background noise was observed. The limits of quantification (LOQ) were determined in the same way by a signal-to-noise of 10. In MSD, SIR mode, the LOD and LOQ for fenarimol in liver samples were of 20 and 60 ng/g, respectively.

The influence of various cone voltage settings on fenarimol fragmentation and the method sensitivity was studied. Fenarimol and the internal standard were analysed by positive electrospray ionization (ES+), with cone voltage varying from 25 to 60 V. Results of positive ES mass spectrum of fenarimol with a cone voltage of 25 and 60 V are shown in Fig. 3. Cone voltage fragmentation was used in order to generate some structural information for fenamirol. However, ethirimol could not be fragmented, even at 60 V.

Extracted ion chromatograms of *m/z* 332 and 210 recorded in the single ion recording (SIR) mode for fenarimol and ethirimol, respectively, are presented in Fig. 4.

The toxicological analysis of our reported fatal case showed concentrations of fenarimol in postmortem samples of 1.9 mg/g in liver, 0.4 mg/g in kidney and 89.0 mg/ml in gastric contents. Due to the high concentrations of fenarimol detected in this fatal case, specially in gastric contents, we had to dilute the extracts before injection, obtaining good results with the highly sensitive method used, which was previously validated in the same conditions.

Fenarimol concentrations obtained in this case may not reflect its concentrations at the time of death since the autopsy was performed 3 days after the probable date of death and the victim was found face down in a puddle, which may facilitate possible postmortem redistribution phenomena. The site- and time-dependent variability of postmortem blood and tissue concentrations, as well as the different

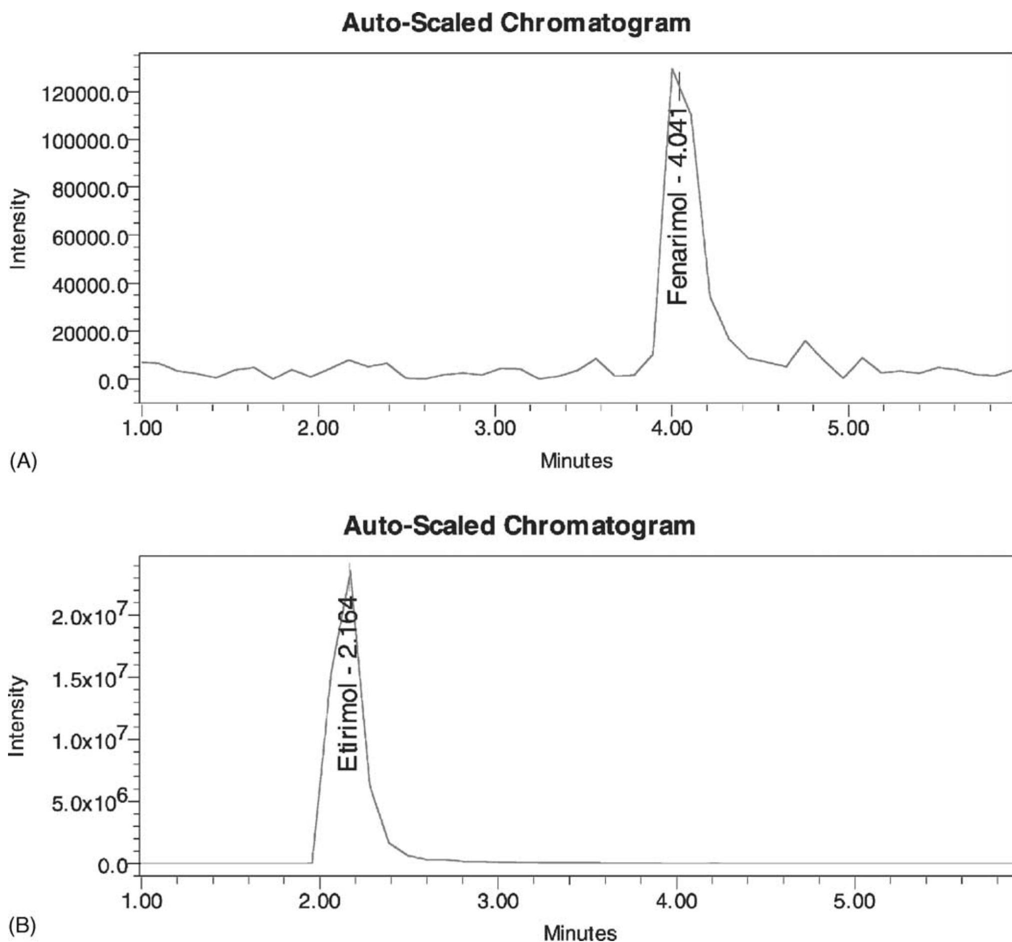

Fig. 4. SIR chromatogram of (A) fenarimol (*m/z* 332 ES+, CV = 25 V) and (B) ethirimol (*m/z* 210 ES+, CV = 25 V).

postmortem redistribution processes possibly involved have been well documented and reviewed [11–18]. Fenarimol is a highly lipophilic pesticide which, associated to putrefactive changes, [15,19] may be submitted to postmortem redistribution, explaining, in some cases, the increased concentrations in some organs and/or tissues. Accordingly, the high concentration obtained in liver samples (1.9 mg/g) in this case may reflect postmortem diffusion from gastric residue to the liver [16,20].

Since no other fenarimol fatal cases were found in the literature, it was not possible to compare these results to those of other authors and therefore to interpret the concentrations found with respect to toxic effects.

5. Conclusion

DAD produces an UV spectra that can be used for the identification of several compounds through spectral matching. However, some UV spectra of different compounds are quite similar and, in these cases, can not be used for positive compound identification. The ability of DAD to provide information on peak homogeneity combined with mass spectral information such as the pseudo-molecular ion of the molecule simplifies the identification of unknowns and increases confidence in chromatographic peak identification. Acquisition in the SIR mode provides increased sensitive and specificity for target compound analysis as well as linear calibration for compound quantification.

The method described here is linear, precise, accurate, sensitive and specific and can be used in routine forensic investigation.

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