Analysis of Endosulfan and Its Metabolites in Human Serum Using Gas Chromatography–Tandem Mass Spectrometry

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Abstract

A new analytical method combining solid-phase extraction and gas chromatography–tandem mass spectrometry (GC–MS–MS) is proposed to determine the insecticide endosulfan as well as its metabolites endosulfan-ether, -lactone, and -sulfate in human serum. Most matrix interferences are avoided using a cleanup step included in the sample treatment and an instrumental technique such as GC–MS–MS, which presents a high sensitivity and selectivity. Recoveries of spiked compounds range between 94.8 and 100.4% and 93.4 and 99.7% at fortification levels of 10 and 30 ng/mL, respectively. The relative standard deviation is lower than 17.6% in all cases, and the limits of detection calculated range from 6 to 19 pg/mL. Serum samples of nine agricultural workers that spray endosulfan into greenhouses in Almería (Spain) and two nonoccupationally exposed people are analyzed, and endosulfan is found in all the samples studied.

Introduction

Endosulfan, also known as (1,4,5,6,7,7-hexachloro-8,9,10-trinorborn-5-en-2,3-ylenebismethylene) sulfite, is a chlorinated insecticide used in many countries because it has less affinity for lipids that are present in most related compounds (1). Endosulfan contains two stereoisomers, α- and β-endosulfan (α- and β-end), that are metabolized and excreted in urine and feces as oxidation products such as endosulfan-sulfate (end-sulfate), endosulfan-alcohol (end-alcohol), endosulfan-ether (end-ether), or endosulfan-lactone (end-lactone). These result from the cleavage of the cyclic sulfite group (2–4) (structural formulas are shown in Figure 1).

Endosulfan presents a moderate toxicity for mammals. It is highly toxic to fish and some bird species and has been shown to have estrogenic effects on humans (5,6). Previous experiments (7) have shown that it can be sampled in air using polyurethane foam. In this study, dissipation processes of endosulfan were studied in different experimental conditions in greenhouses. A total of 7.5% of the initial concentration that was sprayed remained in the greenhouse atmosphere 24 h after application into the greenhouse. Parameters such as vapor pressure, temperature, relative humidity, and even the presence of volatile organic solvents in the emulsifiable concentrates may be influential in the dissipation and decline processes.

Chromatographic methods are widely used for the analytical separation, identification, and quantitation of pesticides in biological samples during one run. In practice, GC using long capillary columns with selective and sensitive detectors such as an electronic capture detector has been one of the most often employed analytical techniques for the determination of pesticides (8–11). Relative retention times are the criteria applied for the identification of chromatographic peaks, but an additional confirmatory technique is also necessary. To this end, the combination of GC and mass spectrometry (MS) has proved its capability for analyzing volatile organic compounds with a high degree of confirmation (12–14). Nevertheless, a low concentration of the target analytes and complexity of various kinds of matrices (such as biological fluids) demonstrate that sensitivity using full-scan GC–MS is not always enough for a solution for analytical troubles. An increase in sensitivity is often performed by using a selected ion monitoring (SIM) mode, but it reduces the quantitative information and the risk of false positives is higher. Furthermore, when biological samples are analyzed, the presence of highly concentrated interfering substances raises background noise, thus obtaining worse results. Recently, the use of GC–tandem MS (GC–MS–MS) has revealed its suitability for the biological monitoring of trace amounts of pesticides and their metabolites (15). The tandem MS is capable of discriminating more efficiently than other MSs between the analyte and matrix signal. Additionally, this detector offers a lot of quantitative information about the target compounds, which improves the trust grade of the results.

A GC–MS–MS method for determining endosulfan and its metabolites in human urine has previously been reported (16). The methodology proposed was applied to the analysis of endo-

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sulfan in the urine of nine agricultural workers that spray pesticides into greenhouses in Almería, Spain. Different amounts of the insecticide and its metabolites were found in all the samples except one. Also, an excretion study of endosulfan in the urine of a pest-control operator that applied the insecticide in controlled conditions was carried out (17). A statistical interpretation of the excretion rate of endosulfan was performed by assuming that it could be described as a first-order kinetic. The constant rate and the half-lives were determined, and it was demonstrated that the α-isomer was eliminated faster than the β-isomer.

Methods for determining endosulfan in human blood were not found in the literature. For this reason, a new method was proposed in this study for determining endosulfan and its metabolites in human serum using solid-phase extraction (SPE) and GC–MS–MS. Serum samples of the pest-control operators whose urine was previously studied were analyzed and the results were compared with the concentration levels found for nonoccupationally exposed people.

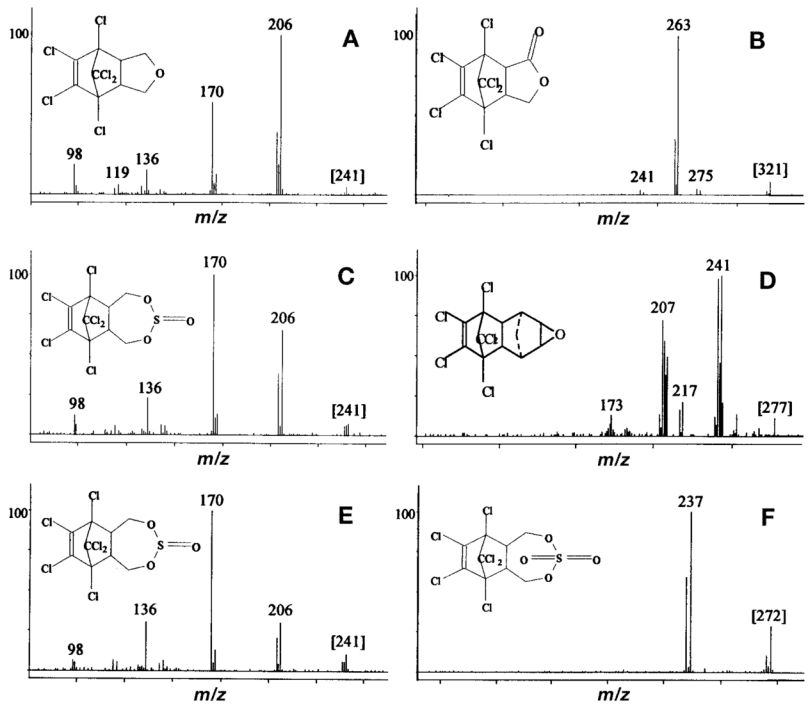

Figure 1. EI-MS–MS spectra and structural formulas: end-ether, A; end-lactone, B; α-end, C; dieldrin, D; β-end, E; and end-sulfate, F.

Table I. MS–MS Conditions*

Compound	Activation time (min)	Retention time (min)	*m/z* range	Parent ion (*m/z*)	Mass defect (mu/100u)	Excitation amplitude (V)	Excitation storage level (*m/z*)
End-ether	6.0–8.0	7.07	80–260	241	–40	83	80
End-lactone	8.0–9.0	8.48	100–340	321	–43	90	141
α-End	9.0–9.5	9.22	80–260	241	–18	83	80
Dieldrin†	9.5–10.0	9.75	50–300	279	–23	2	123
β-End	10.0–11.0	10.44	80–260	241	–18	83	80
End-sulfate	11.0–13.0	11.30	120–290	272	–17	95	119

* Excitation time = 40 µs and isolution window = 2 amu.
† Resonant waveform, u (amu).

Experimental

Chemicals

Pesticide-grade *n*-hexane, diethyl ether, and methanol from Merck (Darmstadt, Germany) were used. Organic-free water was prepared by distillation and then by Milli-Q SP treatment (Millipore Corporation, Bedford, MI).

Analytical standard materials of α-end, β-end, end-ether, end-lactone, end-sulfate, and dieldrin having purities higher than 99% were purchased from Riedel-de-Haën (Seelze-Hannover, Germany).

Stock solutions of an individual compound at 400 µg/mL were prepared in *n*-hexane and stored in a freezer (–30°C). The standard working solutions were obtained by the appropriate dilution of the stock solutions with the same solvent and stored in a refrigerator (4°C). Dieldrin was used as the internal standard at 1 µg/mL in *n*-hexane.

A buffer solution (pH = 7) was prepared by dissolving 1.179 g of KH_2PO_4 and 4.303 g of Na_2HPO_4 (both from Merck) in 1 L of Milli-Q water.

Sep-Pak cartridges for SPE packed with 500 mg of C_{18} were purchased from Waters (Milford, MA).

Equipment

A Saturn 2000 ion-trap MS (Varian Instruments, Sunnyvale, CA) was used. The GC was fitted with a split/splitless programmed temperature injector 1078 operated in the splitless mode and a DB5-MS chromatographic column (30-m × 0.25-mm i.d., 0.25-µm film thickness) (J&W Scientific, Folsom, CA). The ion-trap MS was operated in the electron-ionization (EI) mode, and the MS–MS option was used. The computer that controlled the system had a customized EI-MS–MS library with EI-MS–MS spectra of the target compounds obtained in our experimental conditions. In addition, another commercial EI-MS library was applied (18). The carrier gas used was helium (99.999% purity).

A test-tube shaker with a speed control was purchased from Ika-Works, Inc. (Wilmington, NC).

Sample collection

A 10-mL venous blood sample was drawn with sterile vacuum tubes containing a gel barrier (silicone coated) from nine agricultural workers and two nonoccupationally exposed people under fasting conditions and before a work shift. Serum was obtained by centrifugation for 10 min at 3500 rpm. The samples were immediately frozen and kept at –30°C until they were analyzed. The operators' exposure to the pesticide was assessed by a questionnaire, interview, and medical examination.

Analytical procedure

Serum extraction procedure

A 3-mL aliquot of serum to which was added 90 µL of the internal standard was mixed with 7 mL of the buffer solution (pH = 7) using a test-tube shaker. The mixture was passed through a C_{18} cartridge previously conditioned with 5 mL of methanol and then with 3 mL of the buffer solution (pH = 7) avoiding dryness. In order to carry out a cleanup step, 1 mL of distilled water and 0.1 mL of methanol were added consecutively. The cartridge was dried by passing air through it for 10 min with a vacuum pump. Analytes from the cartridge were eluted using 30 mL of an *n*-hexane–diethyl ether mixture (80:20). The extract obtained was evaporated under a stream of nitrogen without heat and redissolved in 0.5 mL of *n*-hexane. Thus, the extract was ready to be injected into the GC.

GC–MS–MS conditions

A 2-µL aliquot of the extract was injected into the GC with the injector split closed for 1.5 min. The carrier gas flow was set at 36.6 cm/s (150°C oven temperature) and the injector temperature was programmed to begin at 90°C, hold for 0.1 min, rise to 280°C at 200°C/min, and then hold at 280°C for 18 min. The oven temperature was set at 60°C and held for 1.5 min, raised to 220°C at 50°C/min, and then raised from 220°C to 300°C at 5°C/min.

The MS was calibrated weekly. The EI mode had an electron energy of 70 eV; the multiplier voltage was set at 1700 V (1×10^5 of multiplier gain); the A/M amplitude voltage was 3.6 V; temperatures were fixed for trap, manifold, and transfer-line at 200°C, 45°C, and 280°C, respectively; the emission current for the ionization filament was 80 µA; and the automatic gain control (AGC) was switched on with an AGC target set at 3000 counts. The MS–MS conditions are summarized in Table I.

Results and Discussion

Instrumental variables

The GC conditions were optimized to separate endosulfan and its metabolites that were studied with the available column. All of them were properly separated in less than 12 min. The retention times for the target analytes in the selected conditions are shown in Table I.

In the MS–MS mode, the parent ion isolated from the first ionization was selected for each analyte by taking into consideration its *m/z* and its relative abundance (both as high as possible) in order to improve sensitivity. An isolation window of 2 amu was used because the compounds exhibited ion clusters in their MS spectra and wider windows would therefore catch additional neighboring ions and worse repeatability. The AGC target was set at 3000 counts because higher values cause electrostatic interactions between ions in the ion-trap chamber. A nonresonant waveform (collision-induced dissociation, CID) (19,20) was selected for all the compounds except dieldrin, which needed more cleavage energy to obtain a high-quality secondary spectrum. The objective was to generate CID spectra with the parent ions as their molecular peaks (between 10 and 20% of the relative abundance). The base peaks of the secondary spectra were used as quantitation ions. The excitation amplitude was studied for this propose. An excitation storage level (relative to the trapping field that stabilizes the parent ion) was selected for each analyte at the minimum value that would allow for the dissociation of the parent ion. Higher excitation storage values made the ion more stable because of the impossibility of its subsequent breakage to form product ions (secondary spectrum). Moreover, it must be taken into consideration that the excitation storage level is the minimum weight that will be observed in the CID spectrum; therefore, the selection of a value too high may cause a loss of interesting product ions. The excitation time was set at 40 ms when it was necessary to optimize the number of collisions and thus the sensitivity. The EI-MS–MS spectra obtained in the experimental conditions selected are represented in Figure 1.

Extraction variables

The capability of SPE using C_{18} cartridges for the analysis of endosulfan in different biological samples such as human urine versus liquid–liquid extraction has been proven in previous experiments (16,17). An additional cleanup step can be performed to avoid matrix interferences and improve the column efficiency. Dryness must be avoided throughout the conditioning of the cartridge and sample load, but better recoveries were obtained when cartridges were dried before elution. Insignificant differences were found when analyzing human serum or plasma, but the direct extraction of whole blood was discarded because even diluting the samples with buffer solution or methanol caused the C_{18} cartridges to be barred by red globules.

In order to determine of the optimum polarity needed to elute the analytes from the cartridge, various aliquots of a clean serum

Table II. Calibration curves*

Compound	Intercept	Slope	coefficient (%)	Determination P_{lof} (%)
End-ether	0.021	0.156	99.71	0.98
End-lactone	–0.023	0.123	99.64	0.93
α-End	–0.002	0.088	99.02	0.99
β-End	0.006	0.074	99.44	0.98
End-sulfate	3.783	0.203	99.71	0.99

* $n = 3$.

Table III. Recoveries, RSDs, LODs, and LOQs in Serum

	10 ng/mL*		30 ng/mL*		pg/mL	
	Recovery		Recovery			
Compound	(%)	RSD	(%)	RSD	LOD	LOQ
End-ether	98.7	14.8	99.7	13.9	6	26
End-lactone	94.8	17.6	95.9	6.7	19	68
α-End	99.1	14.1	93.4	10.4	14	51
β-End	96.6	7.6	96.6	12.8	15	55
End-sulfate	100.4	14.9	97.4	10.2	10	34

* $n = 5$.

fortified with 30 ng/mL of each compound were passed through different C_{18} cartridges. The analytes were eluted with 50 mL of various solvents (*n*-hexane, diethyl ether, acetone, methanol, dichloromethane, and mixtures of these solvents) that were divided in aliquots of 5 mL collected separately. Quantitation was done using calibration curves obtained with standard solutions of the target compounds in *n*-hexane. The results proved that 5 mL of an *n*-hexane–diethyl ether mixture (80:20) was enough for eluting the analytes from the cartridge (except for the metabolite end-lactone which needed 30 mL to recover 60%).

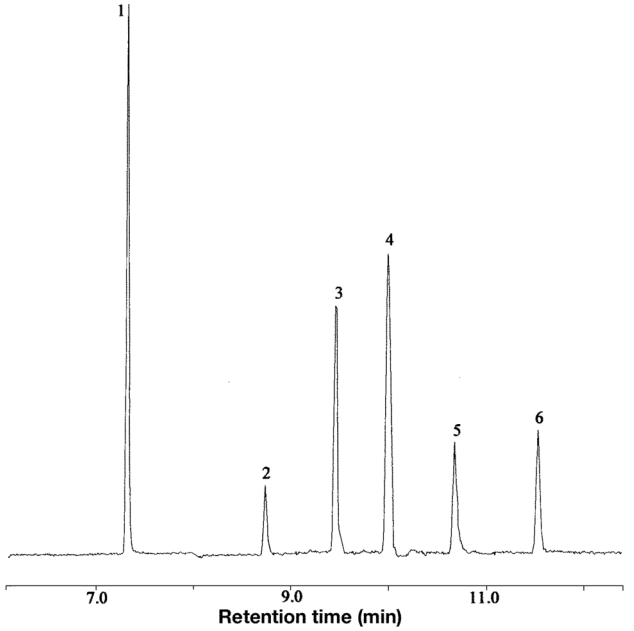

Figure 2. Total ion chromatogram of a human serum sample containing 30 ng/mL of each compound: end-ether, 1; end-lactone, 2; α-end, 3; dieldrin, 4; β-end, 5; and end-sulfate, 6.

Table IV. Concentration* in Serum Samples of Occupationally and Nonoccupationally Exposed People

Sample no.	Last application	Age	Work experience (years)	End-ether	End-lactone	α-End	β-End	End-sulfate	Total
Occupationally exposed									
1	one day	55	40	n.d.†	n.d.	9.39	6.48	n.d.	15.87
2	one day	35	20	n.d.	n.d.	10.31	5.94	n.d.	16.25
3	one day	37	20	n.d.	n.d.	14.54	5.78	n.d.	20.32
4	one day	40	0.6	n.d.	n.d.	3.88	1.68	n.d.	5.56
5	7 days	50	17	n.d.	0.18	12.49	6.86	n.d.	19.35
6	7 days	20	5	n.d.	n.d.	8.34	3.76	n.d.	12.10
7	7 days	51	40	n.d.	n.d.	9.22	3.98	n.d.	13.20
8‡	7 days	47	7	n.d.	n.d.	12.84	5.89	n.d.	18.73
9	7 days	37	25	n.d.	n.d.	4.94	2.77	n.d.	7.71
Nonoccupationally exposed									
10	–	27	–	n.d.	n.d.	4.52	2.66	n.d.	7.18
11‡	–	28	–	n.d.	n.d.	4.43	2.19	n.d.	6.62

* ng/mL.
† n.d., not detected.
‡ Female.

Analytical parameters

The target analytes were searched and identified by their retention time (± 10 s window) and confirmed by comparing their spectra with those stored in the EI-MS–MS library under the same experimental conditions. The search results were scaled to 1000 for best match (identical spectra). The confirmation was considered negative when the match was below a threshold value of 700 or when a signal-to-noise ratio of the chromatographic peak obtained by monitoring the quantitation ion was < 3.

Calibration curves were obtained by using clean serum samples fortified at 0.1, 1, 10, 25, and 50 ng/mL of each compound (the internal standard was 30 ng/mL) and applying the analytical procedure described. In this way, sample and calibration standards are treated in the same manner. A potential matrix effect or recovery deficiency is compensated by normalizing the results obtained for end-lactone. The lack-of-fit test, or P_{lof}(%), (21) was used for three replicates of each concentration in order to check the linearity of the calibration graphs. The values found for the test demonstrated good linearity for all the analytes in the studied range. All of the intercepts were statistically insignificant. The results are summarized in Table II.

The efficiency of the proposed SPE method was assessed. Recovery and repeatability studies were carried out at the fortification levels of 10 and 30 ng/mL (n = 5). The results obtained are shown in Table III. All the compounds presented recovery rates that were between 94.8 and 100.4% at the level of 10 ng/mL and between 93.4 and 99.7% for the samples containing 30 ng/mL. Repeatability expressed as the relative standard deviation (RSD) was lower than 17.6% in all cases. Figure 2 shows a chromatogram of a human serum extract containing 30 ng/mL of each compound.

Table III also summarizes the limits of detection (LOD) and quantitation (LOQ) calculated for the analytes of the proposed method. Ten blank serum samples were extracted and injected. The baseline was integrated at the retention time of the compounds in a zone as wide as the base peak of the analytes at the lowest concentration of the calibration curve. The signal from the blank using the background noise from the chromatogram was evaluated as recommended by IUPAC (22). The LODs and LOQs calculated were in the low parts-per-trillion range. This high sensitivity was reached because of the drastic reduction of background noise. When trace levels of pesticides must be analyzed in dirty samples such as biological fluids, MS–MS improves the results obtained using other MS techniques such as the full-scan or SIM mode.

Applications of the method

The proposed method was applied to nine serum samples from agricultural workers (eight males and one female) previously studied (16) and two samples from nonoccupationally exposed people (one male and the other female). The

workers were between 20 and 55 years old. They were classified into two groups taking into consideration that four of them had applied pesticides at work the day before and the other five sprayed a week earlier. Nonabnormal symptoms were found in the medical examination. The nonoccupationally exposed people were 27 and 28 years old. The results obtained are listed in Table IV.

The α- and β-isomers of endosulfan were found in all of the samples analyzed, including subject No. 7 whose urine did not present pesticides or metabolites above the LOD (16). However, the metabolite end-lactone was detected only once in the agricultural workers' serum studied (0.18 ng/mL). In contrast with the urine sample results, insignificant differences were found between the group of agricultural workers that applied pesticides the day before (a mean of 14.50 ng/mL, standard deviation 6.3) and the people that sprayed pesticides a week earlier (mean 14.25 ng/mL, standard deviation 4.9). Nevertheless, the mean levels of the total endosulfan found in the farmers were significantly higher than the mean level present in the nonoccupationally exposed people group (mean 6.90 ng/mL, standard deviation 0.4). The mass chromatogram obtained for sample No. 5 is shown in Figure 3.

The results obtained revealed that the pesticide could be detected practically intact in human blood, but when human urine was analyzed, a full range of parent compounds and metabolites were usually found. However, the similarity of the results obtained from the serum samples of the two studied groups and the differences found when the urine samples were analyzed (16) seemed to indicate that urine analysis offers information about a recent exposition of the individual and serum analysis shows that information can be attributed to less recent expositions.

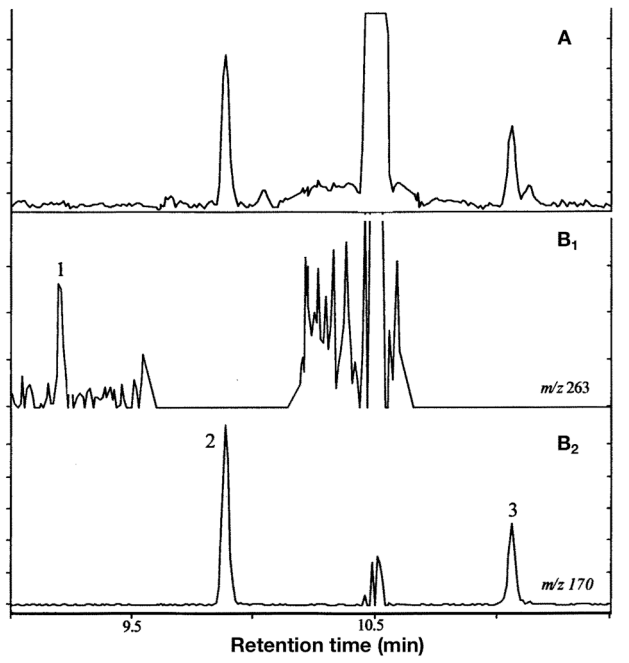

Figure 3. Total ion (A) and mass chromatograms (B_1 and B_2) selecting the quantitation ions of an agricultural worker's serum: end-lactone, 1; α-end, 2; and β-end, 3.

Conclusion

A fast, sensitive, selective, and accurate method for the occupational and nonoccupational analysis of endosulfan and its metabolites in human serum has been proposed. The SPE sample treatment achieved clean extracts that were later measured using GC–MS–MS. When the serum samples of nine agricultural workers were analyzed and the results compared with that obtained for two nonoccupationally exposed people, significant differences were found between both study groups.

Acknowledgments

This research was supported by the European Union (Project SMT4-CJ96-2048 (DG-12-RSMT)) and the Comisión Interministerial de Ciencia y Tecnología (C.I.C.Y.T.) (Project AMB97-1194-CE). This article is dedicated to Dr. Antonio Arrebola Ramírez of the University of Granada, in memoriam.

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Manuscript accepted December 20, 2000.