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Journal of Chromatography B, 713 (1998) 323–330

JOURNAL OF CHROMATOGRAPHY B

Sensitive determination of methomyl in blood using gas chromatography–mass spectrometry as its oxime *tert*.-butyldimethylsilyl derivative

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Received 29 October 1997; received in revised form 17 April 1998; accepted 17 April 1998

Abstract

A sensitive, selective and reliable method was developed to determine methomyl {methyl-*N*-[(methylcarbamoyl)oxy]-thioacetimidate}, a carbamate insecticide in human blood, using gas chromatography–mass spectrometry. Dimethylglyoxime served as an internal standard (I.S.). Methomyl in the blood was converted to its oxime form by sodium hydroxide. The solution made acidic with hydrochloric acid was poured into a column packed with Extrelut. Methomyloxime and I.S. were eluted from the column with a mixture of dichloromethane–ethyl acetate–chloroform (65:25:10), transformed to *tert*.-butyldimethylsilyl derivatives, and analyzed by gas chromatography–mass spectrometry in the electron impact mode. The calibration curves were linear in the concentration range from 1 ng/g to 100 ng/g and 100 ng/g to at least 5000 ng/g. The lower limit of detection was 0.5 ng/g. The absolute recoveries were 72–93% and within-day coefficients of variation were 3.1–5.6% at blood concentrations of 10 and 1000 ng/g. Two practical forensic applications are described.

Keywords: Methomyl

1. Introduction

Methomyl (methyl-*N*-[(methylcarbamoyl)oxy]-thioacetimidate, Fig. 1), is a carbamate insecticide frequently used in Japan. The number of cases of poisoning by this drug ranks second to that of herbicides containing paraquat and diquat [1]. Since methomyl is unstable in heat, alkaline and ultraviolet radiation etc., decomposition of this drug is rapid in soil [2] and metabolism and excretion in vivo is rapid [3,4]. Methomyl also decomposes in the gas chromatography (GC) column [5]. Probably for the above reasons, the lowest value in the blood obtained at autopsy was 0.57 μg/g [6]. When the concentration of methomyl in biosamples decreases greatly due to postmortem changes, the detection of this drug becomes very difficult. Moreover there seems to be no documentation on blood levels of workers using this insecticide. Therefore, methomyl is one of the most difficult drugs to study, in terms of determining the level of the drug in human tissues for purposes of forensic toxicological examination.

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0378-4347/98/$19.00
PII: S0378-4347(98)00198-4

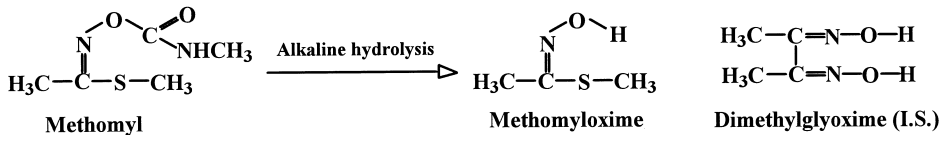

Fig. 1. Structures of methomyl, methomyloxime and dimethylglyoxime (I.S.).

Procedures used for analysis of methomyl in biosamples, include GC [6–8], high-performance liquid chromatography (HPLC) [9–11], and GC–mass spectrometry (MS) [4,12,13]. Among these methods, GC–MS with chemical ionization-selected ion monitoring (CI-SIM) provides the most specific and sensitive approach to detect methomyl in human blood [4,12]. Methomyl was converted to its oxime and analyzed by CI-SIM, as its trimethylsilyl (TMS) derivative. However, the detection limit of these method, 10 ng/g, was not sufficiently sensitive to detect trace levels of methomyl. The reproducibility of the data obtained by CI mode was less satisfactory than in the case of electron impact (EI) mode.

We designed a more sensitive and reliable method to determine concentrations of methomyl in human whole blood, using GC–MS with EI-SIM as its oxime *tert*.-butyldimethylsilyl (tBDS) derivative.

2. Experimental

2.1. Reagents

Methomyl, methomyloxime (methylthioacetohydroxamate), dimethylglyoxime (internal standard, I.S.) and diacetylmonooxime were purchased from Wako Pure Chemical Industries (Osaka, Japan). *N*-(*tert*.-Butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) used for tBDS derivatization and *N*,*O*-bis(trimethylsilyl)acetamide (BSA) used for TMS derivatization were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Dichloromethane, ethyl acetate, chloroform and acetonitrile were of highly purified grade for pesticide analysis and were purchased from Wako Pure Chemical Industries. Other chemicals used were of analytical-reagent grade and were purchased from Wako Pure Chemical Industries. A 120×18 mm I.D. glass syringe with a hypodermic injection needle (1/3) served as an extraction column. Filter paper (No. 2, Advantec Toyo, Tokyo, Japan) was placed at the bottom of the column and the column was packed with Extrelut® powder (Merck, Darmstadt, Germany).

2.2. Biological samples

Whole blood used for control samples was obtained from healthy volunteers and was kept at 4°C until analysis. An anticoagulant was not added to the blood samples. Blank tissue samples were obtained at the time of autopsy and were kept at −20°C until analysis. The blank blood and tissue samples contained no interfering components by forensic toxicological examinations.

Whole blood from the heart and gastric contents obtained from the case 1 victim were kept at 4°C until analysis. The tissue samples (heart, brain, kidney, spleen and lung) collected from the case 2 victim at the time of autopsy were preserved in formaldehyde solution at room temperature for two years. These samples were washed with distilled water and analyzed.

2.3. Standard solution

Methomyl (10 mg) was dissolved in acetonitrile to give a concentration of 1 μg/μl, and was then further diluted to concentrations of 100 and 10 ng/μl with acetonitrile. A standard solution of I.S. was prepared in the same manner.

2.4. Extraction and derivatization

A 1.0-g whole blood sample was weighed and mixed with 1 ml of 2 *M* sodium hydroxide and I.S. solution (50 ng for a sample containing less than 100 ng/g methomyl, 1 μg for a sample containing more than 100 ng/g methomyl) in a 10-ml centrifuge tube. The mixture was kept at room temperature for 1 h. The alkaline solution was made acidic with 1.5-ml of

2 *M* hydrochloric acid and was centrifuged at 2000 *g* (3000 rpm) for 10 min. The supernatant was transferred to another 10-ml centrifuge tube. 0.5-ml of distilled water was added to the remaining precipitate and the mixture was stirred and centrifuged. The combined supernatants were poured into a column packed with 5 g of Extrelut powder, and the column was left to stand for 20 min at room temperature. Methomyloxime and I.S. were eluted with a mixture of dichloromethane–ethyl acetate–chloroform (65:25:10, 15 ml). The eluate was concentrated to ca. 200 μl under a gentle stream of nitrogen at 40°C, and 10 μl (methomyl concentration; less than 100 ng/g) or 20 μl (more than 100 ng/g) of MTBSTFA was added to the solution for tBDS derivatization. The mixture was kept at 60°C for 30 min and a 1-μl aliquot was injected onto the GC–MS system. If the concentration of methomyl was less than 100 ng/g, the reaction mixture was concentrated to 50 μl, and a 1-μl aliquot was injected onto the GC–MS system. For analysis of solid tissues in case 2, a 0.5-g sample was mixed with 1-ml of 2 *M* sodium hydroxide and 50 ng of I.S. solution in a 30-ml centrifuge tube, and the solution was left to stand for 30 min at room temperature. To this solution was added 1 ml of distilled water, then tissue samples were homogenized and kept at room temperature for 30 min, followed by the procedures described above. If the concentration of methomyl was over 5000 ng/g, the sample was diluted with distilled water and then analyzed. A flow scheme of the extraction procedure for methomyl in blood is shown in Fig. 2.

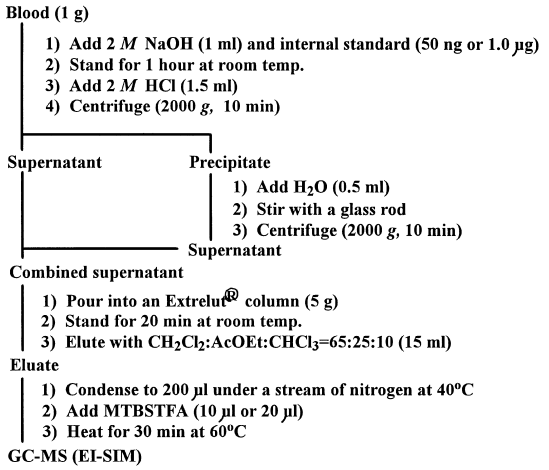

Fig. 2. Flow scheme of the procedure used to extract methomyl from blood samples.

2.5. GC–MS conditions

The apparatus used was a HP5890 Series2 gas chromatograph connected to a HP5989B mass spectrometer (Hewlett-Packard). The column was a Hewlett-Packard crosslinked methyl silicon gum capillary tube of HP-1 (30 m×0.32 mm I.D., 0.25 μm film thickness). The initial temperature of the column was held at 100°C for 2 min, programmed at 20°C/min to 250°C. The injection port and ion source were kept at 250°C. Helium was used as the carrier gas. A splitless injection mode was selected with a valve off-time of 1.0 min. The initial pressure was kept at 30 p.s.i. (1 p.s.i.=6894.76 Pa) (flow-rate; ca. 8 ml/min) for 1 min and in succession the pressure was programmed for constant flow with the flow-rate of 2 ml/min. The septum vent flow-rate was 2 ml/min. EI-SIM mode was selected, and the ions of *m*/*z* 121 and *m*/*z* 162 were used for qualification of methomyl. Since the ion of *m*/*z* 162 showed less interfering peaks than that of *m*/*z* 121 in the chromatogram, it was selected for quantitation of methomyl with the ion of *m*/*z* 287 for the I.S. The relative abundance of *m*/*z* 287 to *m*/*z* 162 was about 1.7.

2.6. Preparation of calibration curves

Two series of whole blood samples were prepared by spiking methomyl to control blood at concentrations of 1, 5, 10, 25, 50, 100 ng/g, each containing 100 ng/g I.S., and at concentrations of 100, 250, 500, 1000, 2500, 5000 ng/g each containing 1 μg/g I.S. These samples were extracted in the same manner as described in Section 2.4. Calibration curves were obtained by plotting the peak-area ratio of methomyloxime-tBDS to I.S.-2tBDS versus the amount of methomyl. As the slope of calibration curve using a blank liver sample was nearly equal that for blood samples, the calibration curve for that whole blood sample was used for quantitation of methomyl in solid tissues preserved in formaldehyde solution.

3. Results and discussion

3.1. Conversion of methomyl to its oxime compound

As methomyl is unstable for heat and easily decomposes in a GC column [5], we attempted to determine methomyl by analyzing methomyloxime, the alkaline hydrolysis product of methomyl. The conversion was completed with 2 *M* sodium hydroxide at room temperature for 1 h. Deproteinization was successful by making the solution acidic with hydrochloric acid, and the oxime converted to its free base was applied to the Extrelut column. As the methomyloxime was much more stable than methomyl itself, reliable analytical results could be obtained, even at a trace level.

3.2. Recovery of methomyl from the Extrelut R column

Among various solid-phase columns, we decided to use an Extrelut column for extraction because dehydration for the following derivatization can be simultaneously achieved. As an eluting solvent, dichloromethane, a mixture of dichloromethane–ethyl acetate, and a mixture of dichloromethane–ethyl acetate–acetonitrile were examined, using various mixing ratios. However, the recovery of methomyl from the column was low, the range being from 43.1 to 52.1%. When we added chloroform to the above solution, the recovery was significantly increased. Using a mixture of dichloromethane–ethyl acetate–chloroform (65:25:10) as an eluting solvent, 96% recovery of methomyl was obtained.

3.3. Derivatization and GC–MS analysis

Noda [4] and Miyazaki et al. [12] analyzed methomyloxime and its TMS derivative by GC–MS, using the CI-SIM mode. When we used their method for our samples, reproducibility of the data by CI mode was unsatisfactory, especially when a trace amount of the drug was analyzed. Therefore, we used the EI-SIM mode for quantitation. The EI mass spectrum of the TMS derivative of the oxime yielded many fragment peaks and sensitivity decreased significantly. We thus attempted to convert methomyloxime to its tBDS derivative. Fig. 3 shows EI mass spectra of tBDS derivatives of methomyloxime and I.S. Only three major ions were observed for each compound, and characteristic ions for tBDS derivatives, $[M-57]^+$, were observed for both methomyloxime and I.S. at m/z 162 and m/z 287, respectively. Sensitivity of methomyloxime-tBDS by EI-SIM was about four-times higher than that of methomyloxime-TMS. As tBDS derivatization for both compounds was completed within 90 min at room temperature and within 30 min at 60°C, we made use of the latter.

3.4. Selection of I.S.

Two ketoxime compounds, diacetylmonooxime and dimethylglyoxime, were examined as an I.S. As the SIM chromatogram with the ion of m/z 287 of dimethylglyoxime-2tBDS showed less interfering peaks than that of m/z 233 of diacetylmonooxime, dimethylglyoxime was used as an I.S.

3.5. Determination of methomyl in blood by GC–MS

Fig. 4 shows SIM chromatograms of tBDS-derivatized extracts from whole blood spiked with 10 ng methomyl and 50 ng I.S. and from blank whole blood. Each peak of methomyloxime-tBDS and I.S.-2tBDS was sharp and symmetrical and was clearly separated with retention times of 4.57 and 7.26 min, respectively. There were no interfering peaks on the chromatograms of blank whole blood.

Since the main objective of this study was to establish a reliable method to determine the low level of methomyl, less than 100 ng/ml, we first made the calibration curve in the concentration range from 1 ng/g to 100 ng/g. However, routine samples taken from the dead victim usually contained over 500 ng/ml of methomyl, hence, correct quantification was difficult using only one calibration curve of low concentration. We therefore made two calibration curves which can be used, depending on the purposes: routine samples in forensic examination should be analyzed using a calibration curve of higher concentration, and samples used to study postmortem changes with methomyl and those used

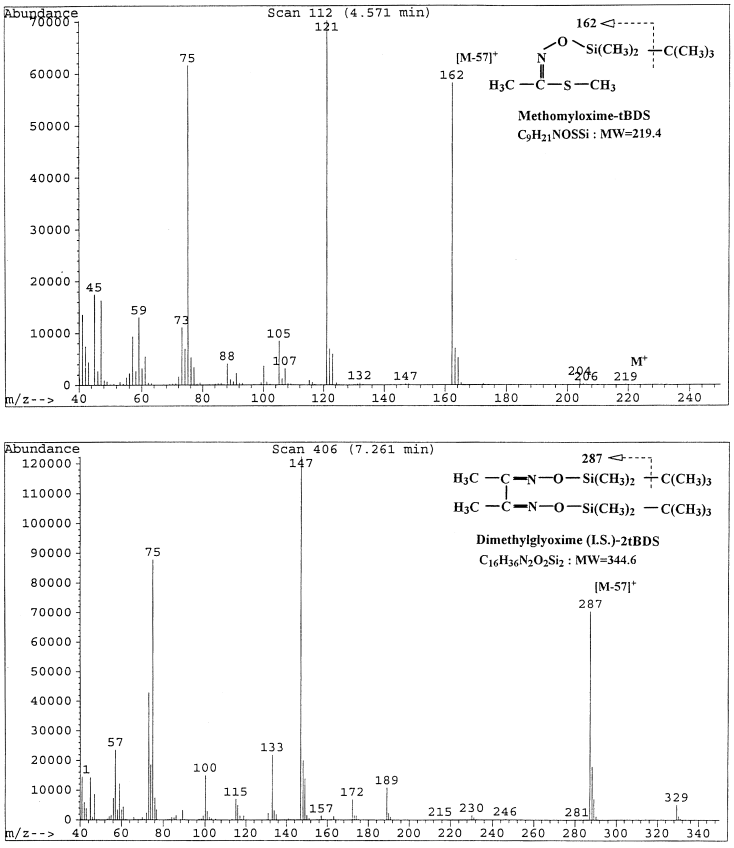

Fig. 3. EI mass spectra of methomyloxime-tBDS and DMGO (I.S.)-2tBDS.

to measure low levels of methomyl in the blood obtained from persons working with this insecticide should be analyzed using a calibration curve of lower concentration.

The calibration curves were linear in the concentration range from 1 ng/g to 100 ng/g and 100 ng/g to at least 5000 ng/g, with correlation coefficients of 0.998 and 0.999, respectively. The lower limit of detection with a signal-to-noise ratio 5:1 was 0.5 ng/g, a value 1/20-times less than that described in the literature [12]. The absolute recoveries of methomyl determined at 10 and 1000 ng/g were 72 and 93%, respectively.

Within-day and between-day precisions were obtained using two different concentrations (10 and 1000 ng/g) by spiking methomyl to blank whole blood. The coefficients of variation (C.V.s) ranged from 3.1 to 5.6% for the within-day and from 4.3 to 5.9% for the between-day precision. The results are shown in Table 1.

These values are sufficiently sensitive and reliable for determining levels of methomyl in human blood, even trace levels.

4. Practical applications

4.1. Case 1

A 62-year-old woman was found dead in the corridor of her home. An empty paper bag of Lannate R 45 and a glass of beer containing blue powder were in her living room. Heart blood and gastric contents were obtained from the victim at the time of forensic examination carried out 12 h after death, and each sample was analyzed in triplicate,

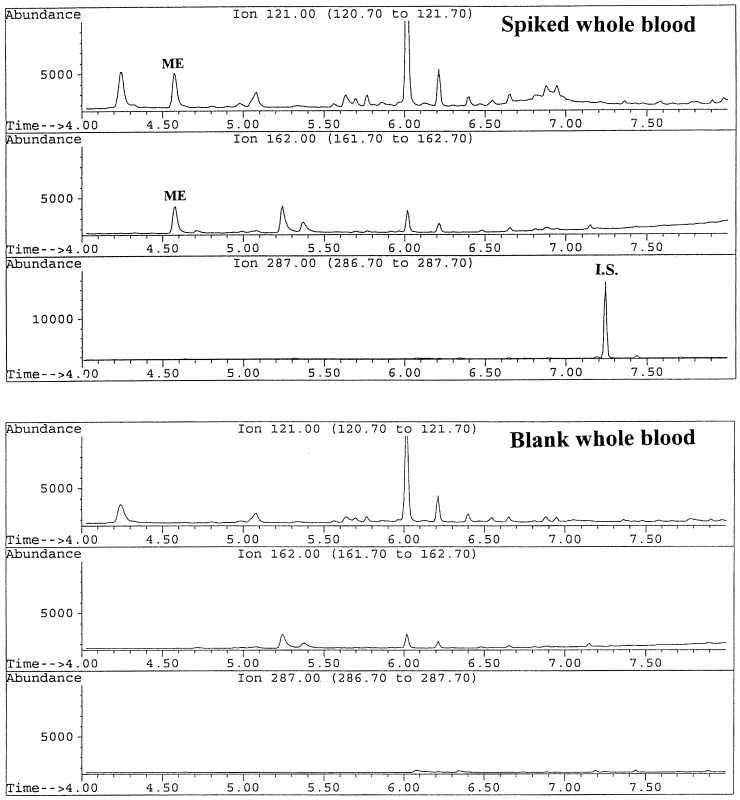

Fig. 4. SIM chromatograms of tBDS derivatized extracts from whole blood spiked with 10 ng/g of methomyl (ME) and 50 ng/g of I.S. and blank whole blood.

using the described method. Blood and gastric contents concentrations of methomyl were 27.7 μg/g and 11.8 mg/g, respectively. Fig. 5 (top) shows SIM chromatograms of blood samples obtained from the woman. Based on the results of blood concentrations of methomyl and data on fatal poisoning, 5.6–63.5 μg/ml, by Tsatsakis and Tsakalof [13,14], the cause of death was diagnosed as acute methomyl poisoning.

4.2. Case 2

A 42-year-old man was found dead at a farm where he worked. Methomyl was qualitatively present in his gastric contents. Solid tissues obtained at the time of autopsy were preserved in formaldehyde solution at room temperature for two years. We used our method to examine these samples. Methomyl was detected in every sample examined; concen-

Table 1
Precision and accuracy for analysis of methomyl in whole blood

Spiked concentration of methomyl (ng/g)	Within-day (n=5)		Between-day (n=5)	
	Found concentration (mean±S.D.) (ng/g)	C.V. (%)	Found concentration (mean±S.D.) (ng/g)	C.V. (%)
10	10.02±0.56	5.6	9.84±0.58	5.9
1000	986.58±30.50	3.1	972.10±41.78	4.3

S.D.=Standard deviation, C.V.=coefficient of variation.

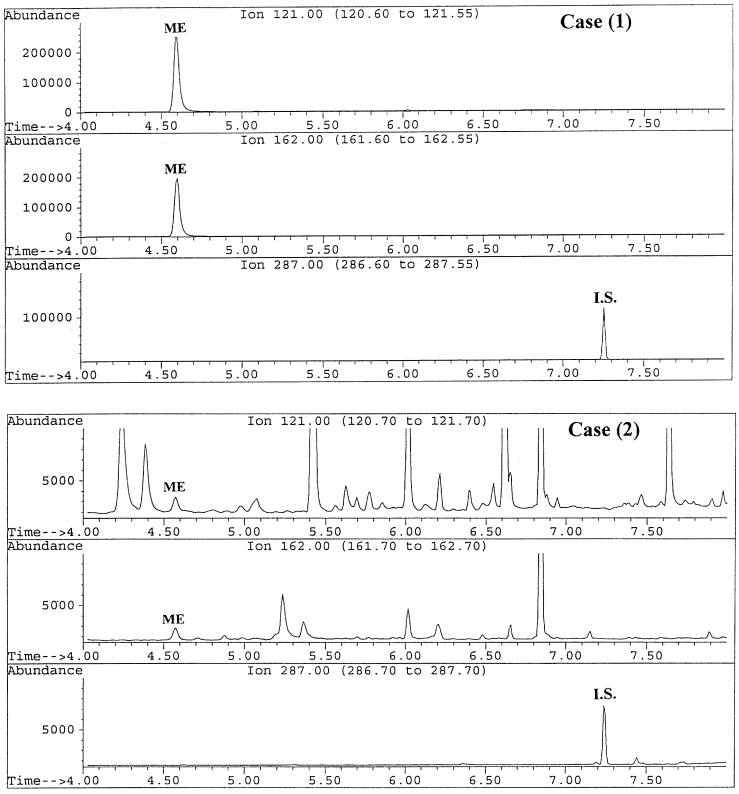

Fig. 5. SIM chromatograms of tBDS derivatized extracts from blood sample of the Case 1 victim and from the heart of the Case 2 victim.

trations of methomyl in the heart, brain, kidney, spleen and lung were 25.3, 22.1, 8.63, 5.66 and 14.9 ng/g, respectively. Fig. 5 (bottom) shows SIM chromatograms of the extract from the heart. Since our preliminary study showed that methomyl concentration in whole blood kept at 25°C decreased rapidly to about 1/30 after one week and less than 1/100 after two weeks, concentrations of methomyl in the tissues in this case were supposedly very high at the time of death, and drastically decreased to trace level during storage of the samples. Although further studies are required in order to confirm the degree of poisoning, the data do provide pertinent evidence of ingestion of this drug.

5. Conclusion

A sensitive, selective and reproducible method to determine the presence of methomyl in human blood was developed using GC–MS. The tBDS derivative of methomyloxime proved suitable for detection by the EI-SIM mode. This method can be used to examine tissue samples for forensic studies.

Acknowledgements

We thank M. Ohara for helpful comments on the manuscript.

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