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Simple, rapid and simultaneous measurement of eight different types of carbamate pesticides in serum using liquid chromatography–atmospheric pressure chemical ionization mass spectrometry

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ABSTRACT

We have developed a method for simultaneous analysis of methylcarbamate pesticides in serum with an acute pesticide intoxication. This is performed by liquid chromatography–atmospheric pressure chemical ionization mass spectrometry. Rapid detection of eight types of methylcarbamate pesticide can be achieved with this method, it only requires an extremely simple pre-treatment of the sample. The specificity of this method is equal to that of gas chromatography–mass spectrometry, and it satisfies the clinical requirements for detection sensitivity and specificity. Although some problems with this analytical method remain to be solved, we consider it to be superior to any other analytical method previously reported.

INTRODUCTION

Environmental pollution is a worldwide problem. Pesticides, a major type of pollutant, are used increasingly in households and on *e.g.* golf courses, as well as in agriculture. The use of carbamate pesticides (CPs) has particularly increased as a result of their broad activity spectrum, high efficiency, and generally low toxicity for mammals. With the increased use of CPs, however, the number of cases of acute CP intoxication has grown.

Acute CP intoxication causes a noticeable decrease in serum cholinesterase (EC 3.1.1.8.; ChE) activity, similar to that observed in cases where organophosphorus pesticides (OPs) are involved. Although serum ChE activity in the cases of OP intoxication is recovered by administering pralidoxime (PAM), ChE activity in the case of CP introxication does not react to the same treatment. Furthermore, PAM administration tends to delay and restrict the natural recovery of ChE activity. For this reason, it is extremely important to immediately determine whether a pesticide intoxication is the result of exposure to OPs or to CPs.

At present, CPs are generally analyzed by spectrophotometry, gas chromatography (GC), high-performance liquid chromatography–ultraviolet spectrometry (HPLC–UV), or gas chromatography–mass spectrometry (GC–MS). Previous reports have noted that spectrophotometry is inferior to the other methods in terms of detection sensitivity and specificity [1,2]. Since most CPs

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are thermally unstable compounds, GC analysis with direct (non-derivation) injection is unsuitable, and the complicated pre-treatment of samples by *e.g.* trimethylsilylation or acylation prolongs the analysis time [3–6]. Although GC–MS is known to have an extremely high specificity, it uses GC for sample separation and hence involves the same problems as encountered in GC analysis [7–10].

HPLC–UV permits analysis under mild conditions, and many CPs absorb at relatively short wavelengths (190–220 nm). In the analysis of CPs in bio-samples of a complicated matrix, this method shows a poor specificity [11–17].

For these reasons, we consider the HPLC–MS method, which combines HPLC separation under mild conditions with the high specificity of MS, to be the most suitable method for analyzing CPs. In this study, we have developed a method for simple and rapid simultaneous detection of eight different types of methyl-CPs (M-CPs) in serum with acute pesticide intoxication, using high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (LC–APCI-MS).

EXPERIMENTAL

Pesticides

We selected eight different M-CPs, each having had an annual consumption in the Saga Prefecture over 100 tons or $100 \cdot 10^3$ liter in 1991. These were: carbaryl (1-naphthyl methylcarbamate), ethiofencarb (2-ethylthiomethylphenyl methylcarbamate), fenobucarb (*o-sec.*-buthylphenyl methylcarbamate), isoprocarb (*o*-cumenyl methylcarbamate), metolcarb (*m*-tolyl methylcarbamate), propoxur (2-isopropoxyphenyl methylcarbamate), XMC (3,5-xylyl methylcarbamate), and xylylcarb (3,4-xylyl methylcarbamate). All M-CPs were purchased from Wako Junyaku (Osaka, Japan). The M-CPs were dissolved in methanol–water (50:50, v/v), and were used as a standard solutions.

Reagents

Distilled water was purified with a Milli-QII system (Millipore, Bedford, MA, USA). HPLC-grade methanol and dichloromethane, and analytical-grade phosphate, ammonium acetate and caffeine were used, all of them were products of Wako Junyaku.

Instrumentation and chromatographic conditions

The HPLC apparatus consisted of an intelligent pump (Model L-6200, Hitachi, Tokyo, Japan), an ultraviolet spectrophotometer (Model L-4000, Hitachi), a sample injector (Model 7125, equipped with 200-μl sample loop; Rheodyne, Cotati, CA, USA), and a column oven (Model 655A-52, Hitachi). A Waters Nova-Pak C_{18} column (15 cm × 3.9 mm I.D., 4-μm average particle size, Millipore) was used. The mobile phase solvents were (A) methanol–water (35:65, v/v) and (B) methanol–water (50:50, v/v). The elution program was set such that the mobile phase (A) was maintained for 5 min and 100% of mobile phase (B) was reached in 25 min at a flow-rate of 1.0 ml/min and at 45°C.

The HPLC setup was connected to a mass spectrometer (Model M-2000, Hitachi) through a non-equilibrium type APCI interface [18]. The interface section was set at a vaporizer temperature of 280°C, a nebulizer temperature of 400°C, an ionization-needle electrode current of 5 μA, and a drift voltage of 240 V. The mass spectrometer was set at a vacuum pressure of $1 \cdot 10^{-4}$ Pa, ion-source slit-width of 500 μm, collector slit-width of 200 μm, accelerated electrical potential of 4 kV, and a secondary electronic step-up tube electrical potential of 1.3 kV. Positive ions in the 0–450 mass range (m/z) were scanned at 8 s intervals.

Serum extract

M-CP standard solutions were added to Lypho-check drug-free serum (Bio-Rad Labs, Richmond, CA, USA) to produce a final concentration of 0.5 μg/ml. A 1.5-ml volume of this sample, 1.0 μg (0.1 ml of 10 μg/ml solution) of caffeine (internal standard), and 1.5 ml of 0.2 M phosphate buffer (pH 7.0) were applied to an Extrelut No. 3 column (Merck, Darmstadt, Germany) and left for 10 min. A volume of 15 ml of dichlo-

romethane was used for elution. The eluate obtained was put through the evaporation process under a steam of nitrogen at 40°C, the residue was dissolved in 150 μl of methanol in water (50:50, v/v). After filtering through a 0.2-μm filter, 100 μl of this solution was injected into the LC–APCI-MS apparatus.

RESULTS

Selection of mobile phase

In LC, a salt is often added to the mobile-phase solvent to enhance the separation efficiency. However, few salts can be used with LC–MS. Of these, acetic acid and ammonium acetate are used most often. To examine the effect of ammonium acetate addition to the mobile phase on the determination sensitivity, we used 0, 10, 20, 50, and 100 m*M* of ammonium acetate buffer (pH 6.8)–methanol (6:4, v/v) as the mobile phase. M-CPs standard solution (500 ng) was injected into the LC–APCI-MS system, and the signal-to-noise (*S*/*N*) ratio determined while monitoring the proton-added pseudo-molecular ions [$(M + H)^+$] and ammonium-added pseudo-molecular ions [$(M + NH_4)^+$] were measured.

In monitoring the $(M + H)^+$ ions, the determination sensitivity in all eight M-CPs and the internal standard decreased markedly after an increase in the ammonium acetate concentration. In monitoring the $(M + NH_4)^+$ ions, no $(M + NH_4)^+$ ions were observed in carbaryl or propoxur even after addition of 100 m*M* ammonium acetate. For the other 7 compounds, the determination sensitivity tends to increase as the ammonium acetate concentration increased. The *S*/*N* ratio was the highest when 100 m*M* of ammonium acetate was used.

In this experiment, the *S*/*N* value was the highest when water–methanol was used as the mobile phase during the monitoring of the $(M + H)^+$ ions. Thus, these conditions were used during the experiments (Fig. 1).

Mass spectra

Fig. 2 shows the mass spectra obtained by injecting 200 ng of each of the M-CP standard solutions under the above mentioned conditions. For every compound, $(M + H)^+$ pseudo-molecular ions of high intensity were registered. These mass-spectra patterns involved cluster ions (*m*/*z* 129, 162) derived from the mobile-phase solvent, 1 or 2 methanol-added pseudo-molecular ions [$(M + nCH_3OH + H)^+$], and a few fragment ions. All the compounds showed relatively simple mass-spectra patterns.

Mass fragmentgrams

Since the $(M + H)^+$ pseudo-molecular ions of the compounds under analysis were generated at high intensity, they were monitored by preparing mass chromatograms. Fig. 3 shows the mass chromatograms obtained by injecting 200 ng of each of the CP standard solutions and 1000 ng of the internal standard (caffeine). The elution of the 9 compounds took about 30 min. Numerous cluster ions derived from water and/or methanol in the mobile phase caused an unstable base line with an ion mass of less than 200.

Detection limits

Using 200 ng of each of the 8 types of M-CP standard solutions, the detection limits with $(M + H)^+$ pseudo-molecular ion monitoring were derived from the *S*/*N* ratio as 3 (Table I). The detection limits were relatively inferior for 4 of the compounds — isoprocarb, metolcarb, XMC and xylylcarb — in which ions with a molecular mass of 200 or below were monitored.

Calibration curves

Calibration curves were prepared by injecting 100 μl of each of the standard solutions containing 1.0, 2.5, 5.0, or 10.0 μg/ml of the 9 compounds, using the above-mentioned determination conditions (Fig. 4). The calibration curves were prepared using the peak area, and the mean value obtained by averaging three measurements of each standard solution was plotted. As showing Fig. 4, the curve is almost linear for concentrations values up to 1.0 μg of pesticides which equals a serum concentration of 10 μg/ml.

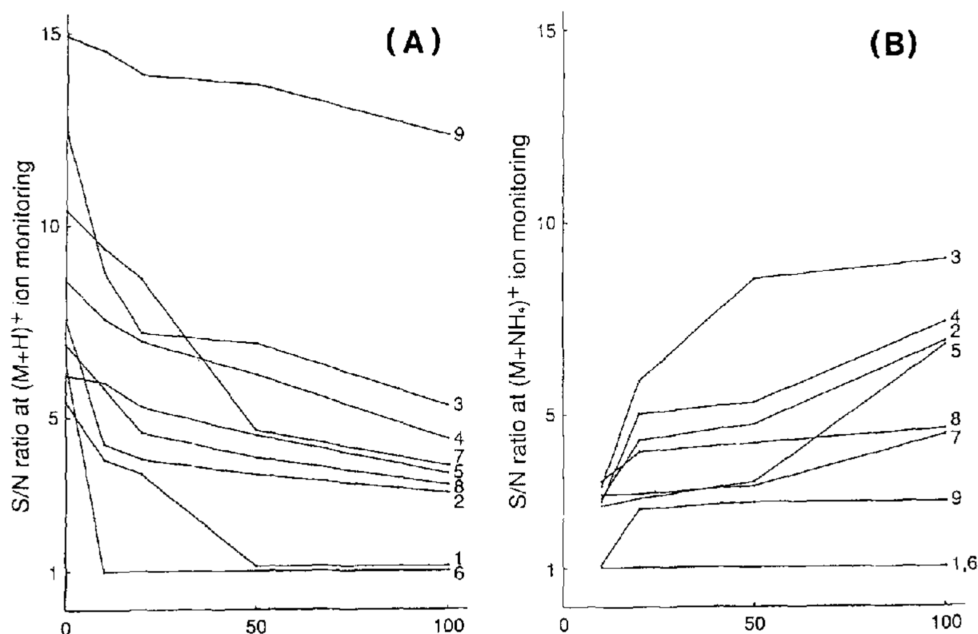

Fig. 1. Effects of ammonium acetate concentration in the mobile phase on determination sensitivity. Mobile phases of 0, 10, 20, 50 and 100 m*M* ammonium acetate buffer–methanol (6:4, v/v) were used. (A) $(M + H)^+$ ion monitoring; (B) $(M + NH_4)^+$ ion monitoring. The *S/N* value was the highest when water–methanol was used as the mobile phase in the $(M + H)^+$ ion monitoring. Peaks: 1 = carbaryl; 2 = ethiofencarb; 3 = fenobucarb; 4 = isoprocarb; 5 = metolcarb; 6 = propoxur; 7 = XMC; 8 = xylylcarb; 9 = caffeine (internal standard).

Recovery test

M-CP standard solutions were added to drug-free serum to produce a final concentration of 0.5 μg/ml. Using this as a sample, a recovery test was performed. The mean recovery for each compound (n = 5) ranged from 93 to 104% (Table II).

DISCUSSION

With the growing use of pesticides worldwide, CPs have been used frequently in recent years. This increase has produced a corresponding rise in the number of patients who ingested CP agents, either by mistake or in suicide attempts. Furthermore, patients who are brought to the hospital for suspected pesticide intoxication are rarely able to identify the agent involved. Diagnosis and treatment depend on rapid and accurate identification of the type of pesticide involved. Accordingly, this study was intended to develop a method to identify CPs in the blood which met such a criteria.

Several different methods are currently used to identify CPs, such as GC, GC–MS, and HPLC [3–13]. Since CPs are thermally unstable substances, GC requires derivation by methods like trimethylsilylation and/or acylation. Complicated pre-treatment of the samples and a long delay before the results are known as negative factors. Furthermore, GC–MS, which offers an extremely high specificity, cannot be used when rapid analysis is required because it uses GC for the separation process. In addition, mass spectra ob-

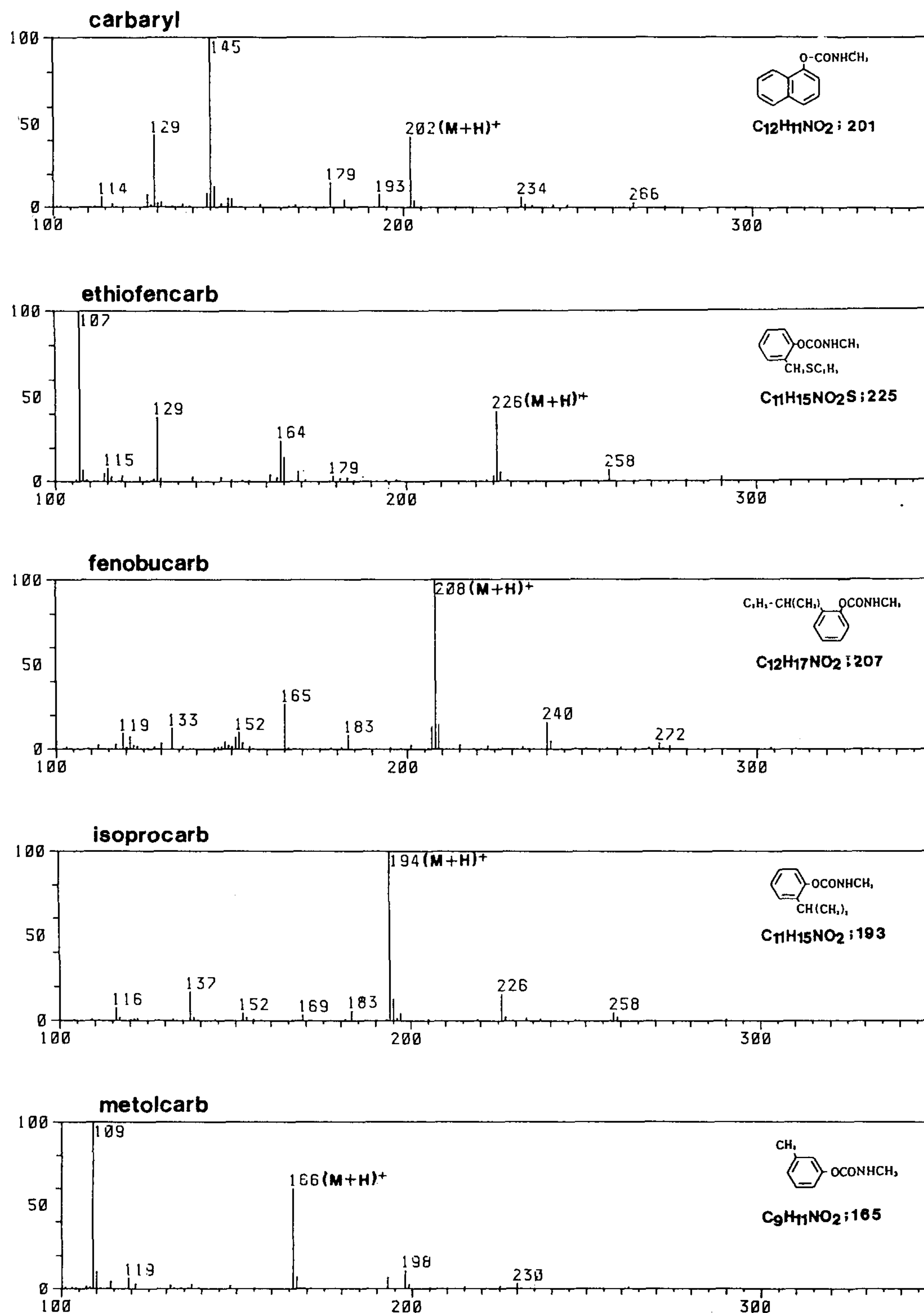

Fig. 2.

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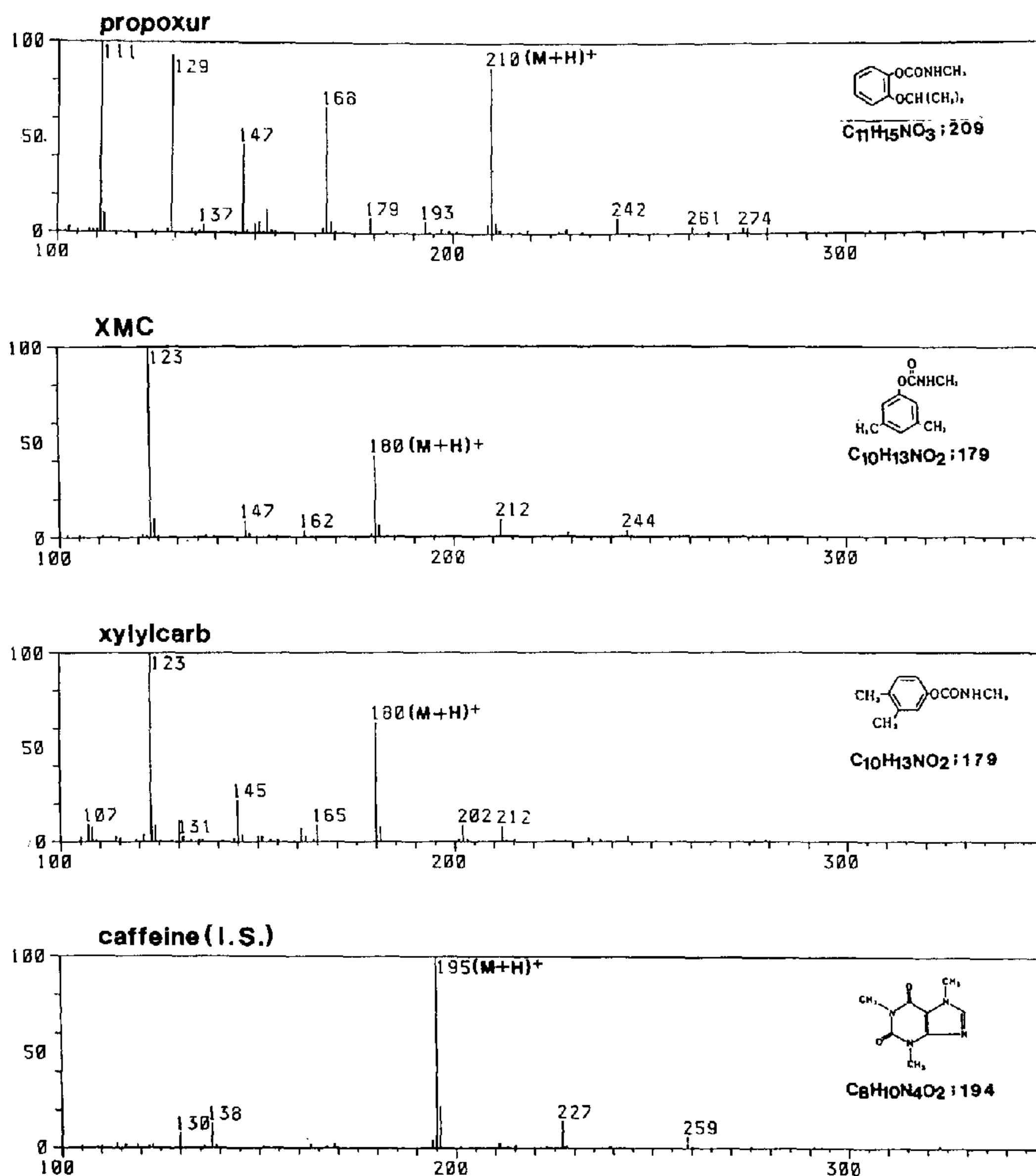

Fig. 2. Mass spectra of 8 methylcarbamate pesticides and caffeine (internal standard). The $(M + H)^+$ ions of high intensity were registered. In addition, $(M + nCH_3OH + H)^+$ ions and several fragment ions were observed. Therefore, this method is a highly specific analysis like GC–MS. LC conditions: column, Nova-Pak C_{18} (15 cm × 3.9 mm I.D., 4-μm average particle size); elution with methanol–water using a 25-min linear gradient from 35% to 50% methanol (v/v) after being kept at 35% for 5 min; flow-rate, 1.0 ml/min at 45°C. APCI interface conditions: vaporizer temperature, 280°C; nebulizer temperature, 400°C; ionization-needle electrode current, 5μA; drift voltage, 240 V. MS conditions: ion-source slit width, 500 μm; collector slit width, 200 μm; acceleration electrical potential, 4 kV; secondary electronic step-up tube electrical potential supply, 1.3 kV; mass range scanned, (m/z) 0–450; scan rate, 8-s intervals.

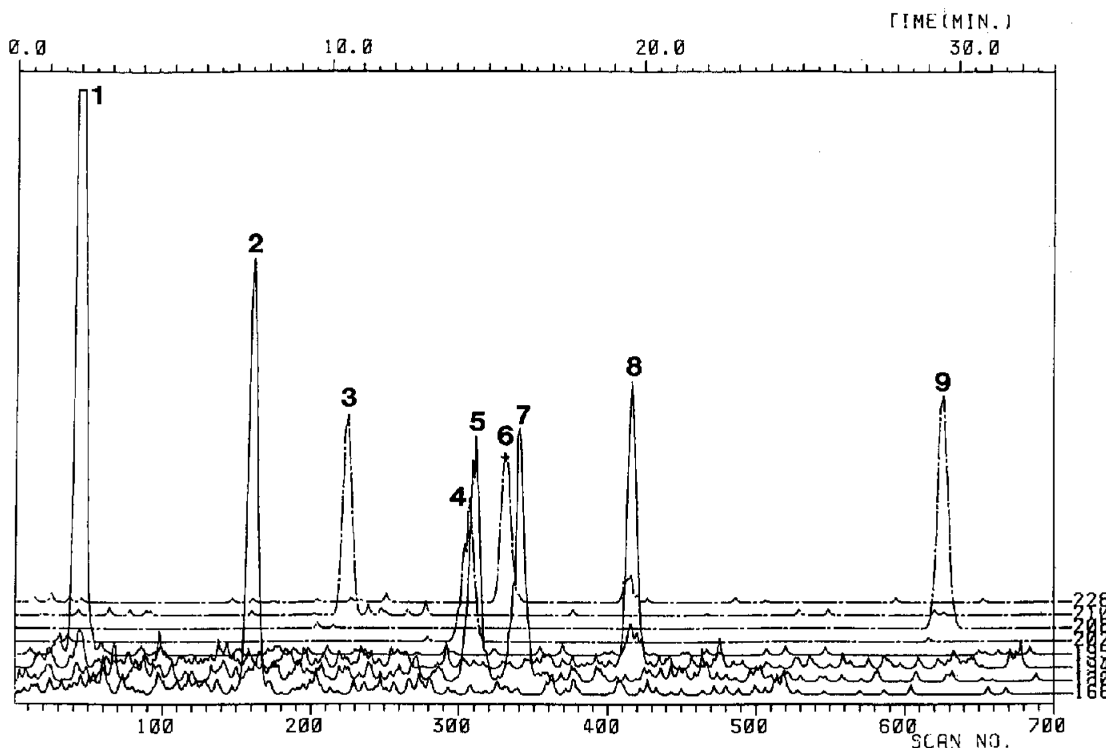

Fig. 3. Mass chromatograms. The elution of the 9 compounds took about 30 min, but the base line was unstable for ions with a mass number of 200 or less. LC, APCI interface and MS conditions as in Fig. 2. Peaks: 1 = caffeine (internal standard) (m/z 195); 2 = metolcarb (m/z 166); 3 = propoxur (m/z 210); 4 = carbaryl (m/z 202); 5 = xylylcarb (m/z 180); 6 = ethiofencarb (m/z 226); 7 = XMC (m/z 180); 8 = isoprocarb (m/z 194); 9 = fenobucarb (m/z 208).

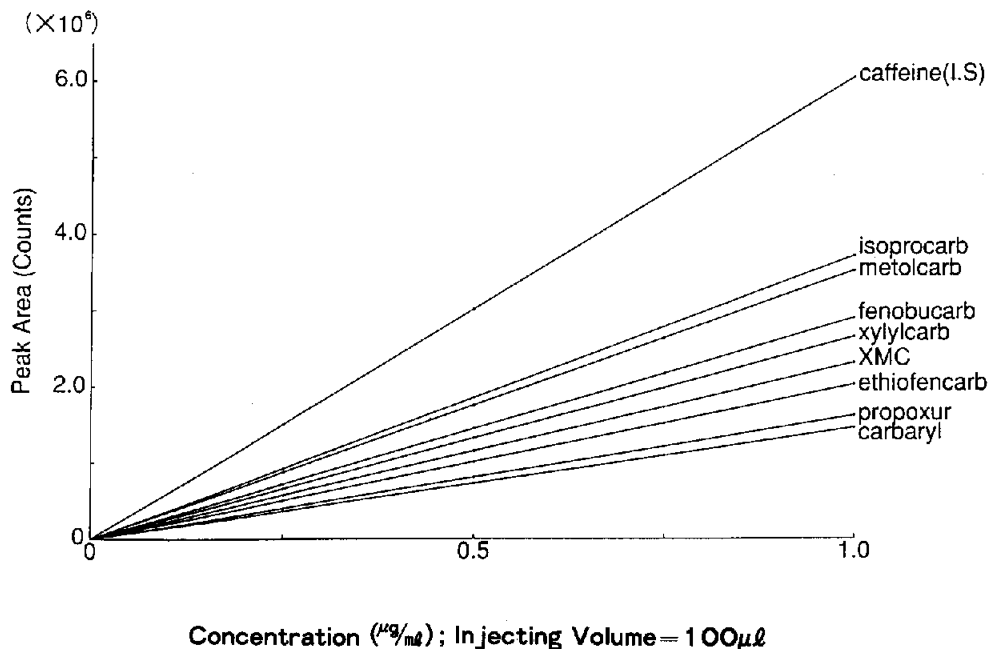

Fig. 4. Calibration curves. A hundred μl each of 1.0, 2.5, 5.0, and 10.0 μg/ml standard solution of the 9 compounds was injected 3 times, and the mean values of the peak areas were plotted. LC, APCI interface and MS conditions as in Fig. 2.

TABLE I

DETECTION LIMITS

The detection limits during mass chromatography were derived using a S/N ratio of 3. LC, APCI interface and MS conditions as in Fig. 2.

Pesticide	Detection limit (ng)
Carbaryl	18
Ethiofencarb	27
Fenobucarb	12
Isoprocarb	60
Metolcarb	42
Propoxure	33
XMC	60
Xylylcarb	60

tained by the electron impact (EI) method are undesirable since the intensity of the molecular ion is extremely low or the ions may not even be observed (Fig. 5). Meanwhile, HPLC–UV is capable of direct analysis without derivation of the samples, and it is the most rapid of the three analytical methods for CP analysis. However, most CPs are absorbed in a narrow wave-length range (190–220 nm). When attempting to determine the complex matrices of bio-specimens, HPLC–UV gives a problem with specificity (Fig. 6).

In this sense, HLPC–MS, which combines the mild separation conditions of HPLC with the excellent specificity of MS, seems to be an ideal analytic method for CP determination. After adding propoxur (PHC) to drug-free serum to produce a final concentration of 0.2 μg/ml, and using the extraction method mentioned above, we analyzed PHC to compare the efficiency of the three methods for qualitative or quantitative analysis — GC–EI-MS, HPLC–UV, and HPLC–APCI-MS (Figs. 5–7, respectively). HPLC–UV produced chromatograms with a PHC peak on the shoulder of a larger peak thought to derive from a substance in the serum (Fig. 6). The method had problems with specificity and sensitivity. In mass spectra obtained by the direct injection method of GC–EI-MS, the low-molecular ionic strength makes fragment-ion monitoring essential for quantitative analysis. For this reason, using GC–EI-MS simultaneous analysis of multiple M-CPs with similar molecular structures will give a low specificity. An approach such as chemical ionization must be used in combination with GC–EI-MS in order to determine the molecular mass of the obtained peak.

In mass spectra obtained by HPLC–APCI-MS, the high intensity of $(M + H)^+$ pseudo-molecular ions made it possible to achieve a high specificity. It confirms that HPLC–MS is a very useful means to simultaneously analyse CPs.

In LC analysis, salt is often added to the mobile phase solvent to enhance the separation performance. Although any highly volatile salt can be used for LC–MS, only a few kinds of salt are currently used. Among them, acetic acid–ammonium acetate buffer solution is most often used for LC–MS analysis because it is easily available, and its usefulness is well recognized [19]. In an experiment that used LC–APCI-MS to examine the effect of addition of ammonium acetate to the mobile-phase solvent on the determination sensitivity for M-CPs, the detection sensitivity of $(M + H)^+$ ion monitoring decreased for all eight M-

TABLE II

EXTRACTION RECOVERIES OF METHYLCARBAMATE PESTICIDES AND WITHIN-RUN PRECISION

Standard solutions of M-CPs were added to drug-free serum to produce a final concentration of 0.5 μg/ml. The sera were used as test samples. Concentrations of the test samples were calculated from the peak areas. LC, APCI interface and MS conditions as in Fig. 2.
($n = 5$)

Pesticide	Mean recovery (%)	C.V. (%)
Carbaryl	98.0	1.0
Ethiofencarb	95.2	2.8
Fenobucarb	93.4	1.2
Isoprocarb	99.5	1.1
Metolcarb	104.0	4.6
Propoxure	97.2	1.7
XMC	97.8	1.3
Xylylcarb	101.0	2.6
Caffeine (I.S.)	98.1	1.3

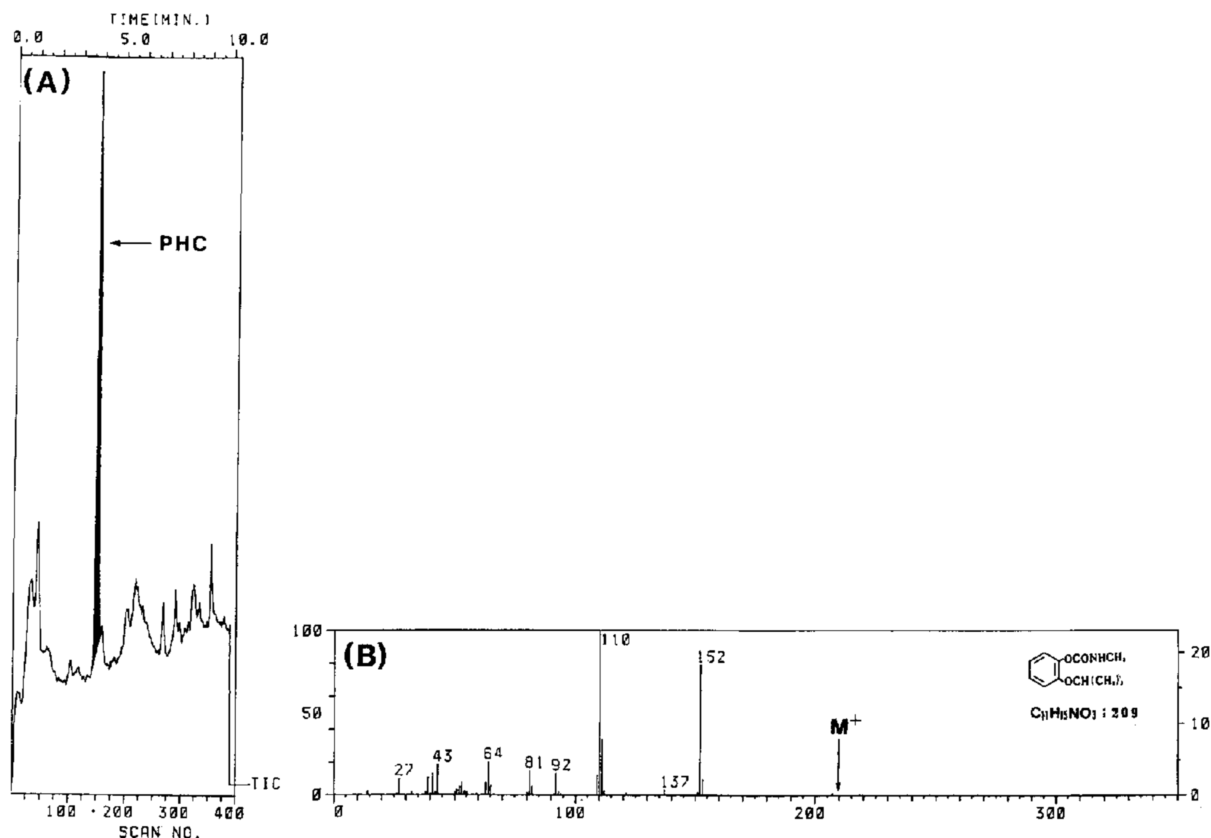

Fig. 5. Determination of propoxur (PHC) in the spiked serum using GC–EI-MS. PHC was added to the drug-free serum to produce a final concentration of 0.2 μg/ml, and the serum was used as a test sample. A 1.0-μl sample adjusted according to the above-mentioned extraction method was injected. In EI mass spectra, the molecular ion indicated very low intensity. Therefore, an approach such as chemical ionization must be used to determine the molecular mass of the obtained peak. (A) total ion current mass chromatogram; (B) EI mass spectrum. GC conditions: column, Ultra-1 (crosslinked methyl silicon gum, 25 m × 0.32 mm I.D., 0.52 μm film thickness, Hewlett-Packard). After being kept at 60°C for 2 min, the temperature of the column was increased to 160°C at a rate of 4 °C/min; injector temperature, 200 °C; separator temperature, 220 °C. MS conditions: ion-source temperature, 180 °C; ion-source slit width, 200 μm; collector slit width, 150 μm; ionization potential, 70 eV; acceleration electrical potential, 4 kV; secondary electronic step-up tube electrical potential supply, 1.2 kV.

CPs (Fig. 1). Ammonium acetate has a higher proton affinity than M-CPs, and the decrease in detection sensitivity caused by the addition of ammonium acetate to the mobile phase seems to be due to an increased efficiency of formation of the NH_4^+ ion relative to that of the CPs^+ ions. Similar results also were reported for LC–APCI-MS analysis of OPs [20]. Accordingly, we thought it to be undesirable to add ammonium acetate to the mobile phase for the analysis of M-CPs.

In the APCI mass spectra of M-CPs obtained using methanol–water as the mobile phase, a high intensity of $(M + H)^+$ pseudo-molecular ions was observed. In addition, several fragmented ions, cluster ions from the mobile phase solvent, and methanol-added pseudo-molecular ions $(M + nCH_3OH + H)^+$ were observed, but the ionic strength was relatively low (Fig. 2). $(M + H)^+$ ions are useful for preparing mass chromatograms, and the fragment ion pattern is useful for identifying peaks. As sum from the GC–MS analysis, this method is highly specific.

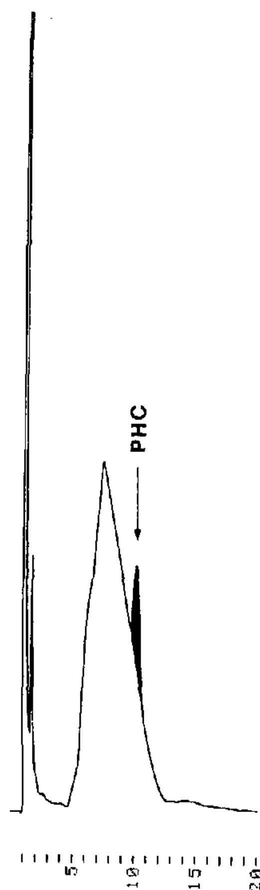

Fig. 6. Determination of propoxur (PHC) in the spiked serum using HPLC–UV. PHC was added to the drug-free serum to produce a final concentration of 0.2 μg/ml, and the serum was used as a test sample. A 100-μl sample adjusted according to the above-mentioned extraction method was injected. The PHC peak rested upon the shoulder of a large peak thought to derive from a substance in the serum. LC conditions: column, Nova-Pak C_{18} (15 cm × 3.9 mm I.D., 4-μm average particle size); mobile phase, methanol–water (4:6, v/v); flow-rate, 1.0 ml/min (45°C); measurement wave range, 210 nm.

In this experiment, it took about 30 min to detect the 9 compounds under the elution conditions employed. However, in the mass chromatograms we obtained by monitoring $(M + H)^+$ ions, the base line was unstable for ions with a mass number of 200 or lower (Fig. 3). The width of the base line fluctuation was larger in the first part of the determination, where the water content in the mobile phase was relatively high, than in the last half where the water content was low. This is due to the effect of the formation of cluster ions derived from water and/or methanol in the mobile phase.

The detection limit of this method was 12–60 ng/ml (Table I). The 4 types of M-CPs for which this method provided a poor sensitivity — isoprocarb, XMC, xylylcarb, and metolcarb — are compounds containing ions with a molecular mass smaller than 200. For these compounds, the base line unstability was caused by the influence of the mobile-phase derived cluster ions. The high noise level resulted in a decrease in the S/N ratio. Accordingly, for high-sensitive LC–APCI-MS determination it is quite important to inhibit the formation of cluster ions with a molecular mass smaller than 200.

The calibration curves were linear up to an injected amount of at least 1.0 μg (Fig. 4). An additionally performed recovery test produced a recovery rate of 93–104% (Table II). These results suggest that this method is capable of complete determination of M-CPs. Though caffeine was used as an internal standard in these experiments, we think a better compound may exist. Further research will focus on selection of an internal standard which is similar to M-CPs in molecular structure and characteristics.

CONCLUSION

We simultaneously analyzed M-CPs in serum using LC–APCI-MS. We confirmed that this method is capable of rapid analysis, and produces a specificity equal to that of GC–MS. The monitoring ions with a mass number of 200 or less gave an unstable base line, and the detection sensitivity was reduced. Future research will need to focus on means of inhibiting the production of cluster ions from the mobile-phase solvent and on the selection of an appropriate internal standard compound.

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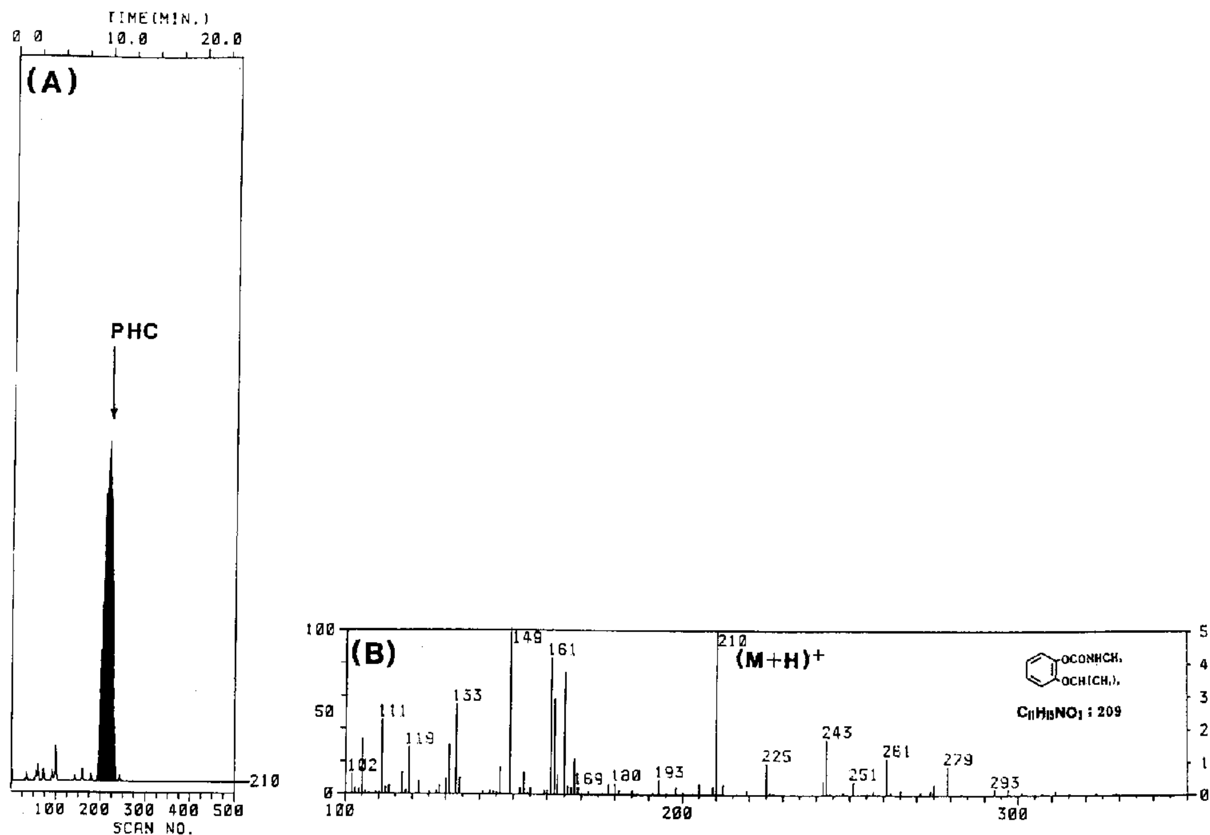

Fig. 7. Determination of propoxur (PHC) in the spiked serum using LC–APCI-MS. PHC was added to the drug-free serum to produce a final concentration of 0.2 μg/ml, and the serum was used as a test sample. A 100-μl sample adjusted according to the above-mentioned extraction method was injected. $(M + H)^+$ pseudo-molecular ion of high intensity was registered. Therefore, highly specific analysis can be performed by monitoring this ion. (A) mass-fragmentgram during $(M + H)^+$ pseudo-molecular ion (*m/z* 210) monitoring; (B) APCI mass spectrum. LC conditions were the same as those shown in Fig. 6, and APCI interface and MS conditions were the same as those shown in Fig. 2.

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