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Screening of organophosphorus pesticides using liquid chromatography–atmospheric pressure chemical ionization mass spectrometry

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ABSTRACT

Screening and identification of organophosphorus pesticides in blood from patients suffering from acute agricultural chemical toxicity were established by a liquid chromatography–atmospheric pressure chemical ionization mass spectrometric method. To determine 21 pesticides, it was necessary to monitor both positive and negative ions. This method could easily screen for 21 organophosphorus pesticides in less than 30 min. By comparison with a gas chromatographic–mass spectrometric method, the chemicals indicated a similar extent of specificity and within equivalent detection limits, thus satisfying clinical requirements completely.

INTRODUCTION

In modern society, many drugs and related compounds are readily accessible. Among these, agricultural chemicals have been comparatively easy to obtain, and these agents continue to be seported in patients with acute drug toxicity as a result of accidental or suicide-intended consumption. It is vital that methods for rapid detection and determination are available to assist in diagnosis and the application of therapeutic countermeasures.

Current analyses of agricultural chemicals involve methods such as gas chromatography (GC) [1–3], high-performance liquid chromatography (HPLC) [4,5] and gas chromatography–mass spectrometry (GC–MS) [6–9]. Among these, GC and HPLC using retention times as criteria are acceptable quantitative methods and are the methods of choice for a number of agricultural chemicals. These methods are reliable, but the identification of an unknown in a matrix that has not been validated may be problematic. GC–MS shows high reliability for both quantitative and qualitative analysis because of the characteristic peaks of compounds in the mass spectra. However, GC–MS has the disadvantage of the decomposition of some thermally unstable substances in the column or derivatization of the specimen during the GC separation.

In recent years, liquid chromatography–mass spectrometry (LC–MS) has been developed to overcome the above problems without losing the specificity of the qualitative analysis of GC–MS [10–20]. In this study, we attempted to detect and screen 21 organophosphorus pesticides (OPs) of compara-

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tively high toxicity by subjecting blood from patients suffering from acute agricultural chemical toxicity to liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (LC–APCI-MS).

EXPERIMENTAL

Pesticides

Twenty-one OPs with consumptions exceeding 10^5 l or 100 tons per year in Saga Prefecture, Kyushu, Japan, were studied: chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate), chlorpyrifos-methyl (O,O-dimethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate), diazinon (O,O-diethyl O-2-isopropyl-6-methylpyrimidin-4-yl phosphorotioate), dichlorvos (2,2-dichlorovinyl dimethyl phosphate), dimethoate (O,O-dimethyl S-methylcarbomoylmethyl phosphorodithioate), dimethylvinphos [2-chloro-1-(2′,4′-dichlorophenyl) vinyl dimethyl phosphate], disulfoton (O,O-diethyl S-2-ethylthioethyl phosphorodithioate), ediphenphos (O-ethyl S,S-diphenyl phosphorodithioate), EPN (O-ethyl O-4-nitrophenyl phenyl phosphorothioate), ethion [O,O,O′,O′-tetraethyl S,S′-methylene bis(phosphorodithioate)], fenitrothion (O,O-dimethyl O-4-nitro-*m*-tolyl phosphorothioate), fenthion (O,O-dimethyl O-4-methylthio-*m*-tolyl phosphorothioate), IBP (O,O-diisopropyl S-benzyl phosphorothioate), isoxathion (O,O-diethyl O-5-phenylisoxazol-3-yl phosphorothioate), malathion [S-1,2-bis(ethoxycarbonyl)ethyl O,O-dimethyl phosphorodithioate], methidathion (S-2,3-dihydro-5-methoxy-2-oxo-1,3,4-thioadiazol-3-ylmethyl O,O-dimethyl phosphorodithioate), parathion (O,O-diethyl O-4-nitrophenyl phosphorothioate), parathion-methyl (O,O-dimethyl O-4-nitrophenyl phosphorothioate), phenthoate (S-α-ethoxycarbonylbenzyl O,O-dimethyl phosphorodithioate), propaphos (4-methylthiophenyl dipropyl phosphate) and pyridaphenthion [O,O-diethyl O-(2,3-dihydro-3-oxo-2-phenylpyridazine-6-yl)phosphorothioate].

Dimethylvinphos was supplied by Sankyo Agricultural Chemical Manufacturing and the other twenty pesticides were purchased from Wako. All chemicals were dissolved in methanol–in water (70:30, v/v) to prepare standard solutions.

Instruments and chromatographic conditions

The HPLC system consisted of an intelligent pump (Model L-6200; Hitachi), an ultraviolet (UV) spectrophotometer (Model L-4000; Hitachi) and a sample injector (Model 7125, with a 200-μl sample loop; Rheodyne). The column was packed with μBondapak C_{18} (30 cm × 3.9 mm I.D., 10-μm average particle size; Waters Assoc.). The mobile phase was methanol–water and elution was performed with a 20-min linear gradient from 70% to 90% (v/v) methanol at a flow-rate of 1.0 ml/min and at room temperature. Methanol was of HPLC grade (Wako) and distilled deionized water purified using a Milli-Q II system (Millipore) was used.

The HPLC system was connected with a mass spectrometer (Model M-2000; Hitachi) via an atmosphere pressure chemical ionization (APCI) interface of the non-equilibrium type [19]. The interface was set with a vaporizer temperature of 250°C, a nebulizer temperature of 400°C, an ionization needle electrode current of 5 μA and a drift voltage of 230 V. The mass spectrometer was adjusted to a vacuum pressure of $1 \cdot 10^{-4}$ Pa, an ion-source slit width of 500 μm, a collector slit width of 400 μm, an accelerated electrical potential of 4 kV, and a secondary electronic step-up tube electrical potential supply of 1.3 kV, and a mass range (m/z) of 0–500 was scanned at 8-s intervals.

Total ion current (TIC) chromatography was applied in the range m/z 200–500 with both UV spectrophotometric and MS detection.

Serum extract

Standard solutions of OPs were added to Lyphocheck drug-free serum (Bio-Rad Labs.) to give a final concentration of 2.0 μg/ml. A 1.5-ml volume of this sample together with 2.0 ml of 0.2 *M* phosphate buffer (pH 7.0) was applied to an Extrelut No. 3 column (E. Merk) and allowed to stand for 10 min. A volume of 15 ml of *n*-hexane–diethyl ether (8:2, v/v) was used for elution. The eluate obtained was evaporated to dryness under a steam of nitrogen at 40°C, the residue was dissolved in 150 μl of methanol–water (70:30, v/v) and 100 μl of this solution were injected into the LC–APCI-MS apparatus.

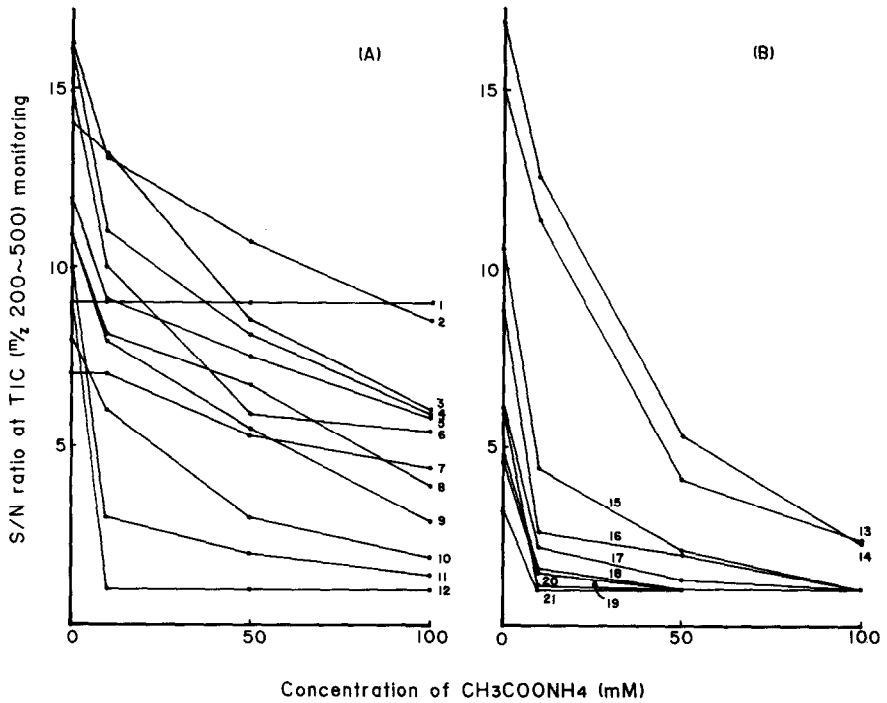

Fig. 1. Effects of ammonium acetate concentration in the mobile phase on the determination sensitivity with TIC monitoring. Mobile phases of 0, 10, 50 and 100 m*M* ammonium acetate–methanol (3:7, v/v) were used. (A) Positive-ion measurement mode; (B) negative-ion measurement mode. 1 = diazinon; 2 = dimethoate; 3 = propaphos; 4 = pyridaphenthion; 5 = ediphenphos; 6 = dimethylvinphos; 7 = IBP; 8 = malathion; 9 = isoxathion; 10 = phenthoate; 11 = dichlorvos; 12 = fenthion; 13 = parathion-methyl; 14 = fenitrothion; 15 = parathion; 16 = methidathion; 17 = EPN; 18 = disulfoton; 19 = ethion; 20 = chlorpyrifos-methyl; 21 = chlorpyrifos.

RESULTS

Selection of ionization mode (positive or negative ions) for measurement

After analyses of standards of the 21 OPs by LC–APCI-MS, the chemicals were classified into three groups: diazinon, dichlorvos, dimethoate, IBP, isoxathion, malathion and propaphos were detectable only with the positive-ion measurement mode, chlorpyrifos, chlorpyrifos-methyl, disulfoton, ethion, EPN, fenitrothion, parathion and parathion-methyl were detectable only with the negative-ion measurement mode and dimethylvinphos, edifenphos, fenthion, methidathion, penthoate and pyridaphenthion were detectable with both the positive- and negative-ion measurement modes. In the last group, methidathion was determined in the negative-ion mode whereas the other five were detected with high sensitivity in the positive-ion mode.

Selection of mobile phase

In LC, a salt is often added to the mobile phase to enhance the separation efficiency. However, as the possible use of the type of salt is limited, acetic acid and ammonium acetate are generally used. To study the effects on the sensitivity of determination of adding ammonium acetate to the mobile phase, the signal-to-noise ratio (S/N) in TIC was measured when 1.0 μg of standards was determined using 10, 50 or 100 m*M* ammonium acetate buffer (pH 7.0)–methanol (3:7, v/v) as the mobile phase.

Ammonium acetate did not affect the detection sensitivity for diazinon, but for the other 20 chemicals marked decreases in sensitivity occured as the ammonum acetate concentration increased (Fig. 1).

Further, except for diazinon and fenthion, the $[M + NH_4]^+$ ion was produced on addition of ammonium acetate to the mobile phase for the other ten chemicals in the determination of positive ions.

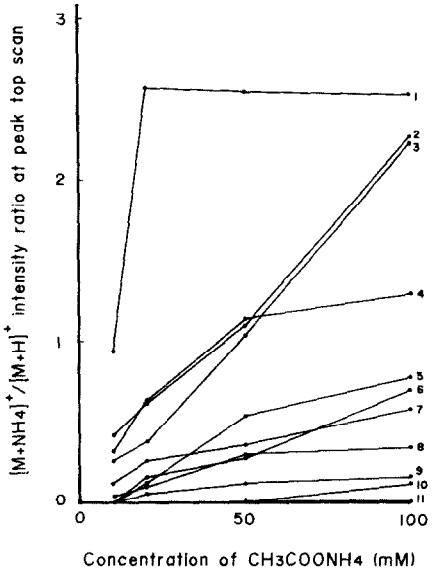

Fig. 2. Effect of ammonium acetate concentration in the mobile phase on $[M + NH_4]^+$ pseudo-molecular ion production. Mobile phases of 0, 10, 50 and 100 m*M* ammonium acetate–methanol (3:7, v/v) were used. 1 = malathion; 2 = dichlorvos; 3 = phenthoate; 4 = dimethylvinphos; 5 = IBP; 6 = ediphenphos; 7 = propaphos; 8 = dimethoate; 9 = isoxathion; 10 = pyridaphenthion; 11 = diazinon.

The ratio of the $[M + NH_4]^+$ intensity to that of the $[M + H]^+$ intensity showed a proportional increase with respect to increase in ammonium acetate concentration (Fig. 2). On the other hand, the $[M + NH_4]^+$ ion was not produced for diazinon. For fenthion, the use of a mobile phase containing 10 m*M* ammonium acetate did not allow the detection of this compound.

Conditions of APCI interface

In LC–APCI-MS, the vaporizer temperature is the limiting factor for the sensitivity of determination. Using methanol–water (70:30, v/v) as the mobile phase, 1.0 μg of the respective OP standard solution was injected and applying TIC chromatography, the *S/N* was derived (Fig. 3). The range of the vaporizer temperature for suitable detection was comparatively narrow; for the determination of positive ions for twelve compounds and negative ions for nine compounds the ranges were 230–250 and 250–270°C, respectively.

Mass spectra

Based on the determination conditions above, mass spectra of 21 OP standard solutions were de-

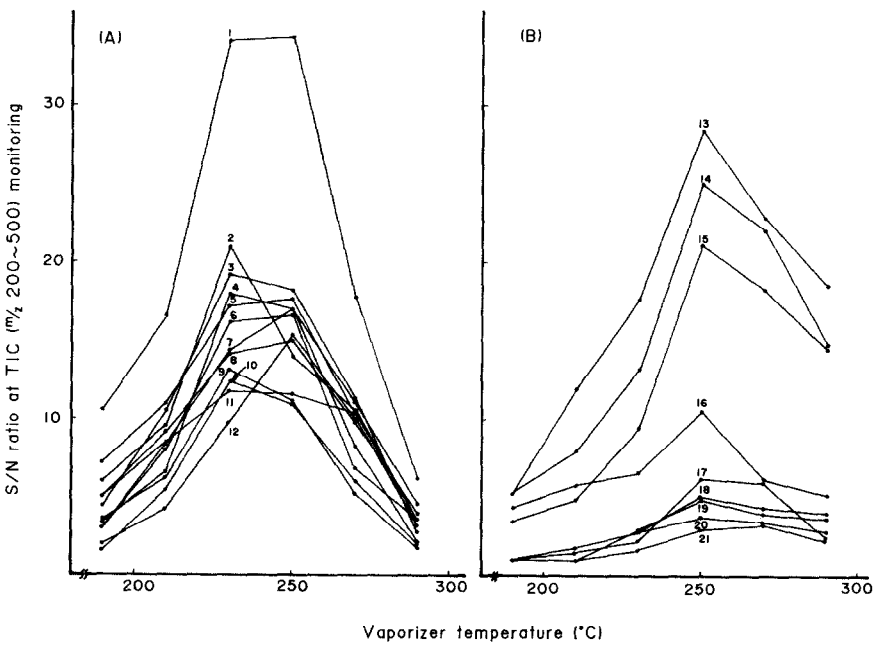

Fig. 3. Influence of the vaporizer temperature on determination sensitivity with TIC monitoring. Mobile phase, methanol–water (70:30, v/v). (A) Positive-ion measurement mode; (B) negative-ion measurement mode. 1 = Dimethoate; 2 = pyridaphenthion; 3 = dimethylvinphos; 4 = dichlorvos; 5 = propaphos; 6 = ediphenphos; 7 = isoxathion; 8 = diazinon; 9 = malathion; 10 = phenthoate; 11 = IBP; 12 = fenthion; 13 = fenitrothion; 14 = parathion-methyl; 15 = parathion; 16 = methidathion; 17 = disulfoton; 18 = EPN; 19 = chlorpyrifos-methyl; 20 = ethion; 21 = chlorpyrifos.

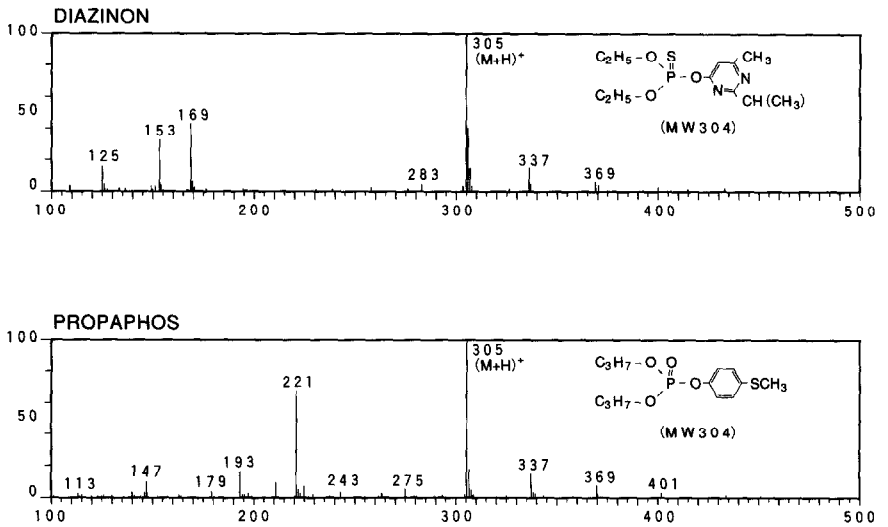

Fig. 4. Mass spectrum (positive-ion measurement mode), showing the positive-ion LC-APCI mass spectra of diazinon and propaphos as examples. These two compounds have a molecular weight of 304 and the $[M + H]^+$ pseudo-molecular ion appeared at m/z 305 as a base peak. However, a fragment ion appeared at m/z 221 with high intensity only with propaphos. Hence the two compounds showed different mass spectral patterns. LC conditions: column, μBondapak C_{18} (30 cm × 3.9 mm I.D., 10-μm average particle size); elution with methanol–water with a 20-min linear gradient from 70% to 90% (v/v) methanol; flow-rate, 1.0 ml/min at room temperature. APCI interface conditions: vaporizer temperature, 250°C, nebulizer temperature, 400°C; ionization needle electrode current, 5 μA; drift voltage, 230 V. MS conditions: ion-source slit width, 500 μm; collector slit width, 400 μm; acceleration electrical potential, 4 kV; secondary electronic step-up tube electrical potential supply, 1.3 kV; m/z range scanned, 0–500; scan rate, 8-s intervals. MW = Molecular weight.

termined. In the determination of positive ions of twelve compounds, $[M + H]^+$ pseudo-molecular ions of high intensity were registered. The $[M + CH_3OH + H]^+$ ion with methanol attached was also observed, although with a low intensity. Moreover, two or three fragment ions, which indicated a simple mass spectrum overall, were indicated (Fig. 4).

For the nine compounds whose negative ions were determined, none produced any $[M - H]^-$ pseudo-molecular ions. The seven compounds disulfoton, ethion, EPN, fenitrothion, methidathion, parathion and parathion-methyl showed fragment ions with monodealkylation replaced by an ether bond. Two or three other fragment ions indicated a simple overall mass spectral pattern. For chlorpyrifos and chlorpyrifos-methyl, $[M - Cl + O; M - 19]^-$ ions showed a standard peak without yielding $[M - H]^-$ pseudo-molecular ions, indicating a mass spectral pattern different from that of the other seven compounds (Fig. 5).

Chromatograms

Chromatograms obtained by injecting 1.0 μg each of the OP standard solutions are presented in Fig. 6A and B. Determination of positive ions was achieved by monitoring the $[M + H]^+$ pseudo-molecular ions. For the negative ions, determination of chlorpyrifos and chlorpyrifos-methyl was based on $[M - Cl + O]^-$ ions, whereas $[M - alkyl]^-$ ions were monitored for the other seven compounds. The total time needed was less than 20 min. However, complete separation for determining the 21 compounds was not attained.

Detection limits

Using standard solutions of the 21 OPs, the detection limits with TIC Monitoring and mass fragmentography were derived for S/N = 3 (Table I). Comparison of the detection limits given by GC, HPLC and GC–MS method showed that the values were slightly inferior with TIC monitoring. Mass fragmentography gave the best results.

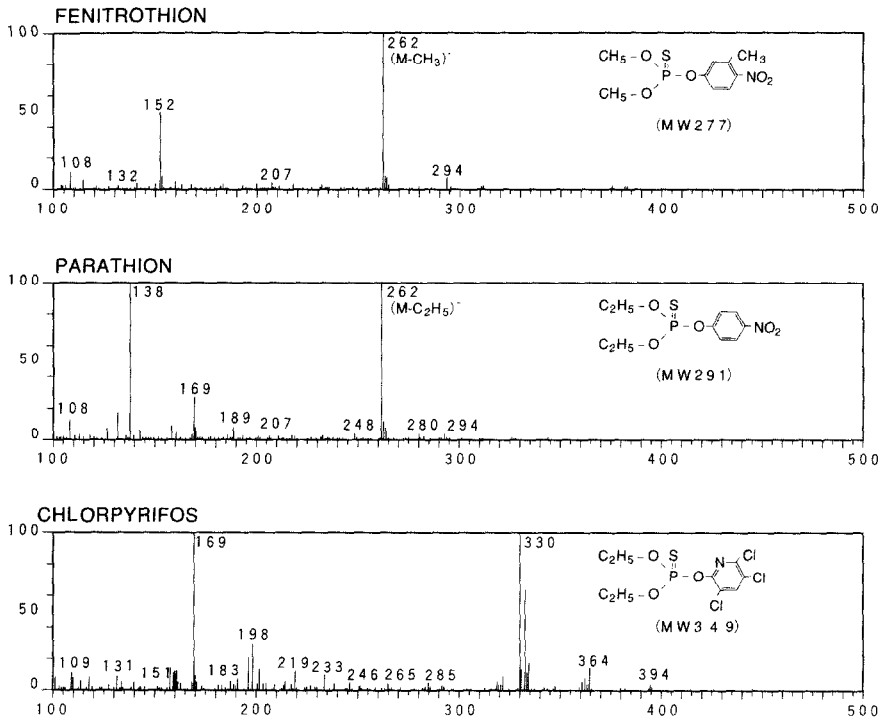

Fig. 5. Mass spectrum (negative-ion measurement mode), showing the negative-ion LC-APCI mass spectra of fenitrothion, parathion and chlorpyrifos as examples. For fenitrothion and parathion, the $[M - alkyl]^-$ fragment ion appeared at *m/z* 262 as a base peak. However, other fragment ions also appeared at *m/z* 152 for fenitrothion and at *m/z* 138 for parathion, so these two compounds showed different mass spectral patterns. In chlorpyrifos, the $[M - Cl + O]^-$ fragment ion appeared as the base peak. LC, APCI interface and MS conditions as in Fig. 4.

Recovery test

OP standard solutions were added to drug-free serum at 2.0 μg/ml. Using this as the sample, measurements were made with UV detection. The mean recovery for each compound ($n = 5$) ranged from 80.3 to 101% (Table II).

DISCUSSION

It is important to perform rapid detection and quantitative analysis and of samples from patients acutely affected with agricultural chemical toxicity in order to be able to take appropriate countermeasures. Many methods for OPs are available, among which GC–MS shows extremely high specificity. However, the use of GC as a separation procedure is impracticable with thermally instable substances and complicated determination procedures involving derivatives are necessary, in addition to other difficulties arising in *in vivo* sample analysis. In recent years, LC—MS has been developed as a practical method that overcomes the disadvantages of GC–MS without losing specificity [10–20].

The 21 OPs studied were classified into three groups that were detectable by only positive-ion measurement, by only negative-ion measurement by both positive- and negative-ion measurement. In LC–MS determinations, the production of positive or negative ions depends considerably on the acidity of the substance in question, and the determination of negative ions for compounds of high acidity has been reported to be appropriate [21]. In the present experiments, we monitored the negative ions for nine compounds and examined the sensitivity. Of these, EPN, fenitrothion, parathion and parathion-methyl contain a nitro group within the molecule. The effects observed are believed to be due to pseudo-acids formed by electron attachment. The

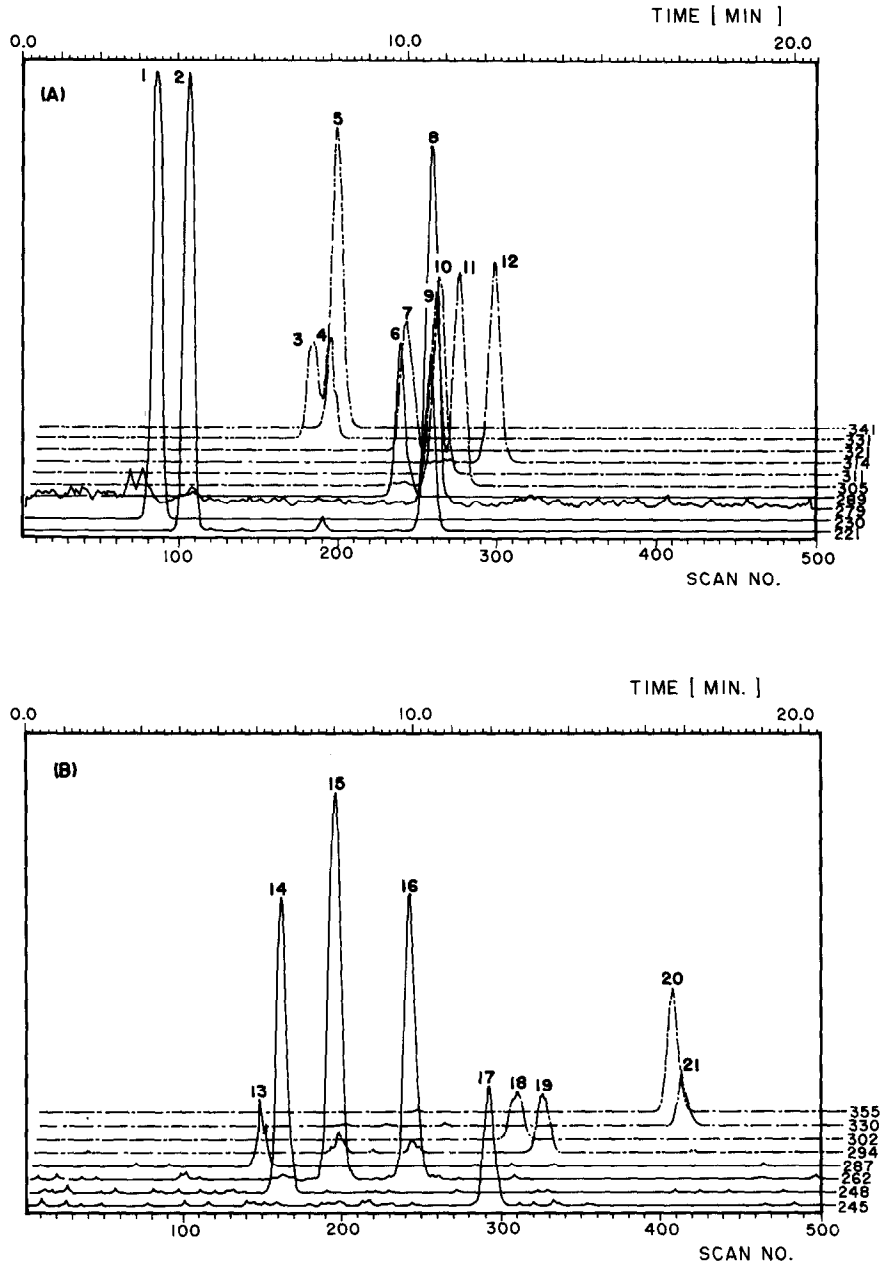

Fig. 6. Mass chromatograms: (A) positive-ion measurement mode; (B) negative-ion measurement mode. LC, APCI interface and MS conditions as in Fig. 4. Peaks: 1 = dimethoate (*m/z* 230); 2 = dichlorvos (*m/z* 221); 3 = malation (*m/z* 331); 4 = dimethylvinphos (*m/z* 331); 5 = pyridaphenthion (*m/z* 341); 6 = IBP (*m/z* 289); 7 = phenthoate (*m/z* 321); 8 = fenthion (*m/z* 279) 9 = propaphos (*m/z* 305, 221); 10 = ediphenphos (*m/z* 311); 11 = diazinon (*m/z* 305); 12 = isoxathion (*m/z* 314); 13 = methidathion (*m/z* 287); 14 = parathion-methyl (*m/z* 248); 15 = fenitrothion (*m/z* 262); 16 = parathion (*m/z* 262); 17 = disulfoton (*m/z* 245); 18 = chlorpyrifos-methyl (*m/z* 302) 19 = EPN (*m/z* 294); 20 = ethion (*m/z* 355); 21 = chlorpyrifos (*m/z* 330).

TABLE I

DETECTION LIMITS

The detection limits during TIC monitoring or mass fragmentography were derived with S/N ratio = 3. LC, APCI interface and MS conditions as in Fig. 4.

Measurement mode	Pesticide	Detection limit (ng)	
		TIC monitoring	Selected ion monitoring
Positive-ion	Diazinon	200	5.0
	Dichlorvos	100	2.0
	Dimethoate	100	2.0
	Dimethylvinphos	200	5.0
	Ediphenphos	200	5.0
	Fenthion	200	20.0
	IBP	200	5.0
	Isoxathion	200	5.0
	Malathion	200	5.0
	Phenthoate	200	5.0
	Propaphos	200	5.0
	Pyridaphenthion	200	5.0
Negative-ion	Chlorpyrifos	1000	50.0
	Chlorpyrifos-methyl	1000	50.0
	Disulfoton	500	20.0
	EPN	1000	50.0
	Ethion	500	10.2
	Fenitrothion	200	2.0
	Methidathion	500	10.0
	Parathion	200	2.0
	Parathion-Methyl	200	2.0

results suggest that the choice between positive- and negative-ion detection also depends on the LC mobile phase conditions, but the detection of both types of ions should be performed when attempting to screen OPs using LC–APCI-MS.

pH adjustment and addition of salt to the mobile phase have sometimes been applied with the aim of enhanceing the separation efficiency in reversed-phase LC. However, as the LC eluate is transferred to the vaporizer via a capillary tube at high temperature, non-volatile salts cannot be used in this method. Although some volatile buffers can be used as the mobile phase in LC–MS, acetic acid and ammonium acetate have generally been adopted and their usefulness is well recognized [22]. From experiments in which the effects of acetic acid and ammonium acetate on the determination sensitivity of OPs were examined, except for diazinon, as the ammonium acetate concentration in the mobile phase increased, a drastic decrease in sensitivity was observed for the other 20 compounds. As ammonium acetate has a higher proton affinity than OPs, the decrease in sensitivity when ammonium acetate buffer is added may be due to the formation of NH_4^+ ions rather than OP^+ ions. It follows, therefore, that it is not appropriate to add ammonium acetate to the mobile phase solvent in the LC–APCI-MS determination of OPs. Although we did not perform the appropriate measurements in this study, other volatile buffers may possibly not have this adverse effect.

The difference in proton affinity between the reagent ion and the analyte has been reported to be a limiting factor the production of pseudo-molecular ions in APCI-MS in general [23]. In this work, even increases in ammonium acetate concentration did not induce the production of $[M + NH_4]^+$ ion for diazinon (for which the determination of positive ions is applied). However, for ten compounds, increases in salt concentration were accompanied by increases in the ratio of $[M + NH_4]^+$ intensity to $[M + H]^+$ intensity (Fig. 2). These findings indicate

TABLE II

EXTRACTION RECOVERIES OF ORGANOPHOSPHORUS PESTICIDES

OP standard solutions were added to drug-free sera to give a final concentration of 2.0 μg/ml as test samples. Measurement was performed using UV spectrophotometric detection, and concentrations of test samples were calculated from peak areas. LC conditions as in Fig. 4.

Pesticide	Mean recovery (%) ($n = 5$)
Positive-ion measurement	
Diazinon	97.2
Dichlorvos	80.3
Dimethoate	81.4
Dimethylvinphos	92.2
Ediphenphos	86.0
Fenthion	85.0
IBP	92.1
Isoxathion	81.0
Malathion	87.1
Penthoate	89.3
Propaphos	91.2
Pyridaphenthion	101.7
Negative-ion measurement	
Chlorpyrifos	85.5
Chlorpyrifos-methyl	88.5
Disulfoton	89.0
EPN	82.4
Ethion	98.6
Fenitrothion	92.0
Methidathion	95.3
Parathion	88.1
Parathion-methyl	93.6

that the production of pseudo-molecular ions in APCI-MS is dependent not only on the difference in the proton affinity of the molecules, but also on the molecular concentration of the reagent gas.

The results of determinations using methanol–water (70:30, v/v) as the mobile phase indicated that the determination sensitivity of LC–APCI-MS is markedly influenced by the size of the droplets of the LC eluate, *i.e.* by the vaporizer temperature. Appropriate temperature ranges for positive- and negative-ions measurements were 230–250 and 250–270°C, respectively. The vaporizer temperature was confirmed to have a great influence on the efficacy of ionization (Fig. 3). From such findings, deciding on the appropriate vaporizer temperature of the LC–APCI-MS method, consideration should be given to the volatility of the mobile phase used (*i.e.*, the organic solvent content in the mobile phase), and not merely to the properties of the analyte. It would be inappropriate to employ an organic solvent concentration that is very different from that in the mobile phase in the gradient elution method. In our experiments, we employed linear gradient elution from 70% to 90% methanol in 20 min. From the results obtained with 1.0 μg of OP standard solution, although elution of the respective compound was completely monitored within 20 min where the determinations were based on positive and negative ions, separation of the mixture was incomplete. However, as these pesticides are rarely multiply present in patients acutely intoxicated with agricultural chemicals, and it is possible to observe the spectral pattern, detection of a particular compound can be achieved. However, given sufficient time for elution to proceed, separation can be enhanced. In some instances, as the peaks which were eluted later from the column showed a broad shape, a decrease is S/N ensued, resulting in a decrease in the detection sensitivity.

The detection limits under the determination conditions employed were 2–50 and 200–1000 ng using selected ion monitoring (SIM) and TIC monitoring procedures, respectively. TIC monitoring is a procedure that cannot be excluded in the establishment of the causative factors in cases of acute agricultural chemical toxicity, and the detection limit of 200–1000 ng is slightly inferior to that of GC–MS [23–25]. However, in our experience, emergency cases admitted with OP intoxication usually register blood concentrations from 0.5 to a few tens of μg/ml, and TIC monitoring is therefore applicable.

Numerous mass spectra of OPs have been reported [17–19, 26–33]. In the APCI-MS determination of positive ions, high-intensity $[M + H]^+$ ions have been observed. Whereas in negative-ion determinations the thermospray method has been used to generate M^- and $[M - H]^-$ ions for OPs, only fragment ions have been noted without the production of $[M - H]^-$ pseudo-molecular ions in the APCI method. In addition, as originally reported, come groups of specific fragment ions of OPs are observed with regard to the detection of unknown peaks, but the specificity is not inferior to that of GC–MS. Further, numerous cluster ions derived from meth-

anol and water in the mobile phase occur at $m/z < 200$. Therefore, TIC chromatograms in the low-mass range show an unstable baseline, resulting in a decrease in S/N. This is the reason why we monitored the TIC chromatogram in the m/z range 200–500. By avoiding the production of cluster ions in such a manner, it is possible to monitor TIC chromatograms at even lower mass range. In other words, it is a possible means to enhance the detection limit.

CONCLUSIONS

We have attempted to screen OPs in blood by an LC–APCI-MS method. Its detection ability was comparable to that of GC–MS. Using TIC monitoring, our method, which is inferior to GC–MS analysis, was found to be a useful approach for determinating substances in patients with acute agricultural chemical toxicity. SIM was equal to other methods. With efforts to decrease cluster ions derived from the mobile phase components and by using columns of smaller inner diameters, we could decrease sample dilution by the mobile phase within the column and therefore procure sharp peaks for more efficient detection. Enhanced detection sensitivity can be further expected with the present method.

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