Sensitive High-Performance Liquid Chromatographic Analysis for Toxicological Studies with Carbaryl

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Carbaryl and its degradation products could be detected in water and in serum at levels as low as 0.5 ng/mL (or 5% full scale responses at 0.005 aufs) by HPLC analysis using a 3-μm C18 Ultrasphere column on a Beckman HPLC System (Beckman, Inc., San Ramon, CA). Preparation included a single extraction with methanol after application of samples to 1.0 mL of C18 solid-phase extraction columns. Advantages of this method include high sensitivity, analysis time of 10 min after a single extraction, and high recovery. Sensitivity was maintained whether the carbaryl or its degradation products or metabolites were provided in water or in serum. This HPLC method would be useful for toxicological studies in which detection of low concentrations of carbaryl were needed.

INTRODUCTION

Carbamate insecticides are widely used today, with over 55 million pounds produced annually in the United States alone (Marshall, 1985). These agents are used as contact and systemic insecticides on crops used for food and feed and may, as well, be applied topically to food-producing animals for control of external parasites (Smith, 1987; Roberts, 1986). Although carbamates are inhibitors of acetylcholinesterase as are the organophosphorus esters, carbamate insecticides are degraded more rapidly and are generally considered to have a lower order of toxicity in animals (Murphy, 1986). These factors have contributed to the widespread use of carbamates since their commercial introduction in the 1960s.

There has been recent concern, however, that carbamate insecticides are not as free of toxicological effects as once suggested. For example, subchronic neurotoxicity has been reported after long-term exposure to carbaryl used as a household insecticide (Branch and Jacqz, 1986). This concern has reemphasized the need for detection methods of even minute quantities of these insecticides, their metabolites, and their degradation products in food, feed, water, and body tissues and fluids.

Chromatographic techniques are currently used for detection of carbaryl. The most sensitive method reported to date has used HPLC for detection of carbaryl and its metabolites at levels down to 5 ng/mL (Ward et al., 1987). This paper describes a simple extraction followed by HPLC analysis on a highly efficient 3-μm column that increased sensitivity for detection of carbaryl and metabolites into the picomole range. This procedure proved useful for detection of carbaryl in water and serum and, therefore, has potential value in toxicological evaluations after low-level exposures.

METHODS

Reagents. Carbaryl and its metabolites (7-hydroxycarbaryl, 6-hydroxycarbaryl, 4-hydroxycarbaryl, 5-hydroxycarbaryl, α-naphthol, methylolcarbaryl, 1,6-dihydroxynaphthalene, 1,5-dihydroxynaphthalene, and 1,7-dihydroxynaphthalene) were gifts from Drs. Allan Scarborough and Richard Heintzelman, Rhone-Poulenc Agricultural Chemicals, Research Triangle Park, NC. All HPLC reagents were Burdick and Jackson high-purity solvents obtained from Baxter Healthcare, Muskegon, MI. Solid-phase extraction (SPE) columns of octadecylsilane (C18) bonded silica gel were from Baker Analytical, Phillipsburg, NJ.

Chromatography. HPLC was performed by using a Beckman 344 system (Beckman, Inc., San Ramon, CA) with UV-vis detector at 210 nm and sensitivity [absorbance units full scale (aufs)] from 0.005 to 0.2. The flow rate was set at 0.5 mL/min of solvent (methanol/water at 51:49). Dilution of sample was decreased by decreasing all extra column volume (i.e., the tubing) to a minimum. The HPLC column (Ultrasphere, Beckman), 4 cm, 3-μm C18 octadecylsilane, was preconditioned by allowing this solvent to flow through for 2 h before application of the first sample and 5 min between samples. The total time to complete the chromatographic analysis of each sample was 10 min. Carbaryl and metabolites for preparation of standard curves and internal standards were weighed on an analytical balance (Cahn 29 automatic electrobalances, sensitivity 0.1 μg) and dissolved in methanol to prepare stock solutions of 1.25 or 2.5 μg/mL. Standard curves were prepared by using dilutions of these stock solutions (0.5, 5, 25, 100, 125, 250, 500, 1250, and 2500 ng/mL methanol), which were evaporated and reconstituted in methanol at 10× concentration before 5 μL was applied to the HPLC column.

Experimental Samples. Serum samples were obtained from rats and from chickens given carbaryl 5 mg/kg iv. They were prepared for HPLC using 0.2 mL of filtered (0.45 μm) serum pipetted onto a 1.0 mL SPE C18 column. These columns were first preconditioned by washing with 2 mL of methanol followed by 3 mL of distilled water. After samples were added, the column was washed with 2 mL of water and then extracted with 0.5 mL of methanol. Extraction was followed by evaporation and reconstitution of the sample with 100 μL of methanol. Five microliters of the reconstituted sample was injected on the HPLC column. Pond water was spiked by adding 10 μg of carbaryl to 100 mL of water. Dilutions were made with pond water so concentrations analyzed ranged from 1.0 to 100 ng/mL. One-milliliter samples were pipetted onto a 1.0-mL preconditioned SPE C18 column, washed with 2 mL of distilled water, eluted with 0.5 mL of methanol, evaporated to dryness, and reconstituted to 250 μL. Five microliters of the reconstituted sample was applied to the HPLC column.

RESULTS AND DISCUSSION

Standard Curves for Carbaryl and Metabolites. Retention times for carbaryl and six of its metabolites are presented in Figure 1. Each compound was clearly separated by the conditions of analysis, and all compounds were eluted from the column in less than 9 min. The highly efficient 3-μm column causes less dilution of sample than columns used in other studies (Ward et al., 1987; Duck and Woolias, 1985). Decreasing column volume and extra

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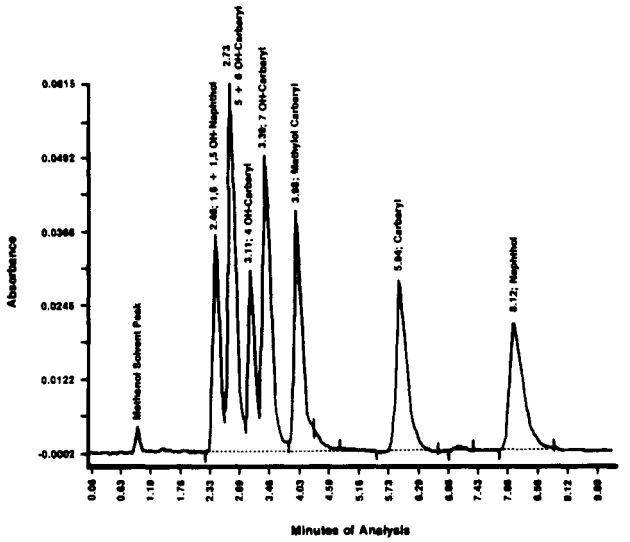

Figure 1. Chromatogram of carbaryl and metabolites as presented by the printer attached to the HPLC. Although sensitivity was set at 0.05 aufs, the computer automatically adjusts to include the total peak heights of metabolities that provided absorbances slightly greater than 0.05. A sample containing 125 ng/mL each was prepared as described under Methods and 6.25 ng in 5 μL applied to a 3-μm C18 Ultrasphere column. The time (in minutes) at which each peak was eluted from the column is listed beside the peaks identified.

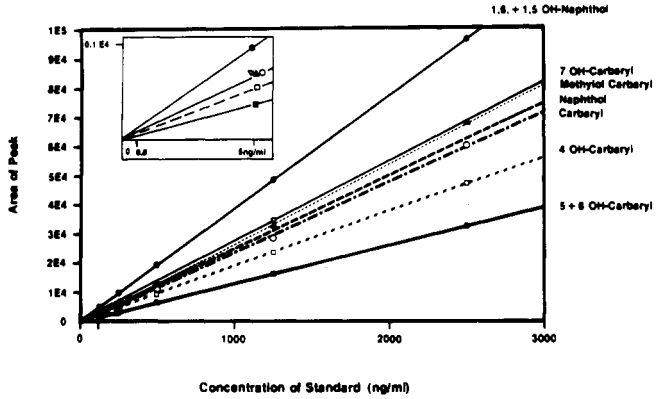

Figure 2. Plot of area under the peak relative to concentrations of carbaryl and its metabolites. Areas of the peaks are expressed as multiples of 10^4–10^5 absorbance area units as calculated by the program for integration on the Beckman 344 HPLC system (1E4 = 1×10^4 absorbance area units, 2E4 = 2×10^4 absorbance area units, 1E5 = 1×10^5 absorbance area units). Concentrations were linear when 5 μL of a 10× concentrate of solutions with original concentrations of 0.5–2500 ng/mL were applied to the column. The insert on the figure expands the lower part of the curve. The curves for 7OH carbaryl, methylolcarbaryl, naphthol, and carbaryl were overlapping at the lower part of the curve and are represented by the solid line marked with ▽▲○. Each line is the average of 16 replications. Standard deviations were less than 5% of the average area obtained at a certain concentration.

column volume, as done in this study, decreases the volume needed to provide for elution of sample peaks, thereby increasing resolution and sensitivity of detection (P. Campbell, Beckman Instruments, personal communication). Areas of the peak obtained for carbaryl and each of the metabolites were linear with concentration of the solution from which they were extracted (Figure 2). The signal to noise ratio was reduced by using ultrapure solvents and by controlling detector threshold and rate of acquisition of data. Precolumn extraction also contributed to low signal to noise ratios when experimental samples were used.

Carbaryl in Pond Water. An example of a chromatogram obtained after pond water to which carbaryl had been added 3 days previously is shown in Figure 3. The same single degradation product (α-naphthol) in the same proportion appeared on five replicates of this experiment. In the example, naphthol was present in concentrations of 51.75 ng/mL when carbaryl was added at 100 ng/mL. The total recovery of carbaryl and metabolite from pond water 3 days after the addition of carbaryl was 99.75%. For each of the five replicates the recovery of carbaryl and naphthol was greater than 99%. The methanol solvent peak and an unknown peak also appear on the chromatogram of pond water. This unknown peak, which appeared at 1.8 min, also appeared in unspiked pond water. Carbaryl and naphthol peaks only appeared on chromatograms that were spiked with carbaryl (Figure 3, insert). Detection of carbaryl was linear with extracts from solutions of concentrations as low as the 1 ng/mL tested in this experiment, indicating that this method would be sensitive for the parts per billion quantities of this insecticide that may be used for toxicological studies on carbaryl in water supplies.

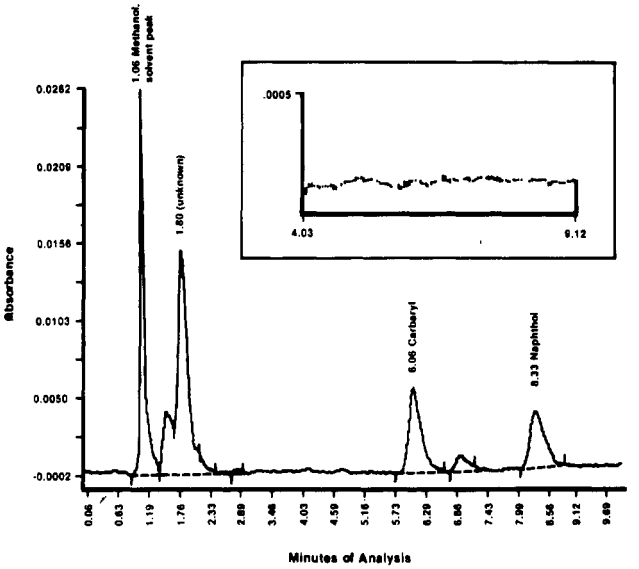

Figure 3. Chromatogram of a methanol extract of pond water containing carbaryl, 100 ng/mL, 3 days after the insecticide was added. Quantities of carbaryl and α-naphthol contained in the 5-μL injection volume were 0.98 and 1.03 ng, respectively. The unknown peak at 1.80 min also appeared in chromatograms of pond water to which no carbaryl had been added. The time (in minutes) at which carbaryl and naphthol were eluted from the HPLC column are listed beside their peaks. A chromatogram of pond water not containing carbaryl is included as an insert.

Carbaryl in Serum. Carbaryl could be detected in serum from rats and chickens after the serum had been applied to an SPE column and extracted with methanol. Sample preparation was relatively easy; 12 samples could be done in 30 min. When serum extracts were applied to the HPLC column, areas under the peaks of chromatograms produced were linear for samples containing between 0.5 and 2500 ng/mL of carbaryl. The peak areas were within 5% of those seen when equivalent concentrations of carbaryl were detected in pond water, and the slope of the concentration curve was identical with that shown for carbaryl in Figure 2.

Serum concentrations of carbaryl and the metabolite methylolcarbaryl measured 3, 20, and 120 min after administration of 5 mg/kg to rats and chickens are presented in Table I. The clearance of carbaryl in the chicken was especially rapid, which may contribute to the relative resistance of this species to toxicities induced by this carbamate insecticide (Cranmer, 1986; Ehrich et al., 1990).

Table I. Concentration of Carbaryl in Serum from Rats and Chickens after Intravenous Administration[a]

species	time after administration, min	concn, mg/mL carbaryl	concn, mg/mL methylolcarbaryl
rat	3	1508 ± 104	not detected
	20	619 ± 146	not detected
	120	91 ± 76	not detected
chicken	3	1323 ± 100	399 ± 99
	20	208 ± 31	247 ± 71
	120	6 ± 3	35 ± 91

[a] Carbaryl 5 mg/kg iv. Results presented as mean ± SE, $n = 5$ (rats) or $n = 6$ (chickens).

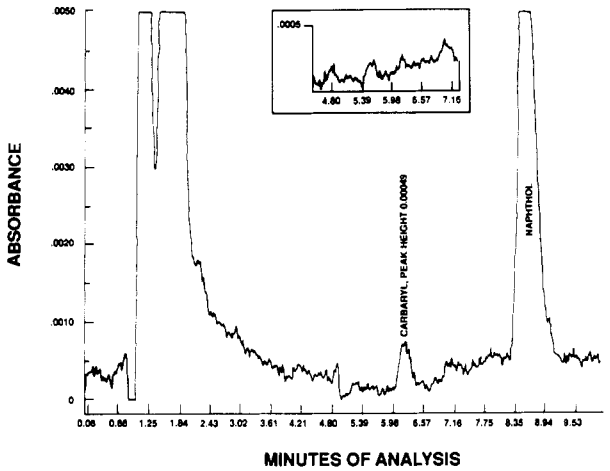

Figure 4. Chromatogram of rat serum containing 1.03 ng/mL carbaryl. The sample was prepared as described under Methods, with carbaryl in 0.2 mL of serum contained in 100 μL of methanol, and 5 μL (0.013 ng) applied to HPLC column. Naphthol (1.0 ng) was used as internal standard. A chromatogram of serum from an untreated rat is provided as an insert.

These studies indicate that low quantities of carbaryl could be detected in water and biological samples. The studies of Ward et al. (1987) provided detection sensitivity at 4 times baseline noise, with estimation that solutions containing 5 μg/mL were near the lowest concentration for which his method was useful. In his procedure, 5 μL of a 20× concentrate (0.5 ng) was applied to a 5-μm reverse-phase column and water–acetonitrite used as the mobile phase. As shown in the example presented as Figure 4, the method described here could identify peaks in serum when significantly less than this amount of preextracted carbaryl-containing sample was applied to a 3-μm reverse-phase HPLC column eluted with methanol–water. Absorbance of carbaryl in extracts containing 0.5 ng/mL was 5% of 0.005 aufs. No peaks with absorbance greater than 0.0004 were seen on the chromatogram of serum samples that did not contain carbaryl at time points between 4.80 and 7.16 min (Figure 4, insert).

The method for detection of carbaryl described here was similar to that of Ward et al. (1987) and Duck and Woolias (1985) in the use of reverse-phase HPLC, UV detection, and a single organic solvent as mobile phase. Several factors were, however, different in the present study which would contribute to the increased sensitivity noted. For example, the volume of solvent for elution of carbaryl was decreased. This decrease in the dilution of sample was accomplished by shortening extra column tubing to a minimum and by use of a 3-μm rather than a 5-μm reverse-phase column. This results in increased peak height on the chromatogram. In addition, the preextraction of samples with methanol on SPE columns and the use of ultrapure solvents would contribute to increased sensitivity in the present study. Also, the data system used allows for excellent signal to noise ratios and permits computer enhancement of the chromatogram (Campbell, personal communication).

HPLC methods other than ours and the ones described above used fluorescence detection to obtain sensitivities to 10 ng/mL (Lawrence and Leduc, 1978; Krause, 1985). Preparation of samples included derivatization and column extraction. These procedures take considerable time without providing the sensitivity of our procedure or that of Ward et al. (1987). Other methods, which use gas chromatography or spectroscopy, are not as sensitive as methods using HPLC for carbaryl detection (Bezuidenhoul and van Dyk, 1981).

In addition to sensitivity, the HPLC method for carbaryl detection developed in our laboratory takes relatively less time for analysis than other methods described. Twelve samples, for example, could be prepared for chromatography in 30 min, with 10 min needed for HPLC analysis of each. Therefore, the method is economical as well as extremely sensitive. Like the procedure described by Ward et al. (1987), our method could also be used for quantitation of carbaryl and its metabolites and degradation products. The method described, therefore, has potential for a variety of applications for studies in environmental toxicology.

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Registry No. Water, 7732-18-5; carbaryl, 63-25-2; methylolcarbaryl, 132179-02-3; 7-hydroxycarbaryl, 32263-67-5; 4-hydroxycarbaryl, 5266-97-7; 6-hydroxycarbaryl, 32263-74-4; 5-hydroxycarbaryl, 5721-72-2; α-naphthol, 90-15-3; 1,6-dihydroxynaphthalene, 575-44-0; 1,5-dihydroxynaphthalene, 83-56-7; 1,7-dihydroxynaphthalene, 575-38-2.