Analysis of Benomyl by Liquid Chromatography/Time-of-Flight Mass Spectrometer and Its Occurrence in the Environment

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Benomyl, one of the known endocrine disrupting chemicals, was analyzed to understand the fate in the nature. Water, sediment and biota samples are acidified to hydrolyze benomyl into carbendazim then followed by extraction and concentration. The concentrations of carbendazim in the samples were determined by liquid chromatography/time-of-flight mass spectrometer. Analysis data showed that certain amount of carbendazim was accumulated in sediment. On the contrary, no sign of accumulation in biota was observed probably due to the increased degradation rate *in vivo*. It is, however, that no one can claim carbendazim is not harmful to biota, since carbendazim may give a negative effect against organisms at the point of intaking.

Keywords : Benomyl, Carbendazim, LC, LC/MS-TOF.

Introduction

Due to the high toxicity and slow degradation rate, a lot of organochlorinated pesticides are prohibited and replaced by relatively mild pesticides such as organophosphorous or carbamate pesticides. However, the nature of thermal lability of carbamate pesticides gives a great limitation in determination of trace level residues. Direct analysis using gas chromatography (GC) is not recommendable since the carbamate pesticides sample can be broken down in the hot column during the analysis. A few complicate derivatization methods have been applied to overcome the thermal lability.[1] Liquid chromatography (LC) has been used as an alternative of overcoming the thermal lability of carbamate pesticides since LC analysis do not use heating process. Although LC can avoid the thermal decomposition of carbamates, low sensitivity and complicate sample preparation according to the selected detector prevent it from wide use for the determination of trace level residues of these compounds in environmental samples such as food or drinking water. Our group determined the concentration of methyl (1-butylcarbamoyl)-2-benzimidazolecarbamate (benomyl) by liquid chromatography/time-of-flight mass spectrometer (LC/MS-TOF) to study its fate in the environment. LC/MS (TOF) turned out to be an excellent analysis instrument in the determination of carbamate pesticides since it has not only very low detection level but it can avoid thermal decomposition.

Benomyl has been widely used as a systematic fungicide for on variety of food crops and ornament plants.[2] Besides its carcinogenic activity, it has been known for numerous years that chronic, subchronic, and acute administration of benomyl to rats and mice result in male reproduction damage.[3] The acute systematic toxicity of benomyl is very low with a rat LD_{50} of 10 g/kg approximately.[4] In contrast, a single dose of 100 mg/kg is capable of eliciting a testicular lesion.[5] It has been speculated that benomyls metabolite, carbendazim, cause testicular toxicity by the same mechanism of which they act as fungicides.[6]

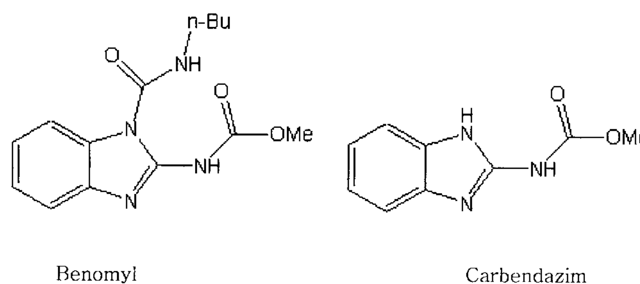

Figure 1. Benomyl carbendazim.

Benomyl is rarely soluble in water and rapidly degrades to carbendazim in the environment (Figure 1). The degradation rate is dependent on the pH, temperature, and moisture. High pH and temperature accelerate the break down rate of benomyl. The benomyl metabolite, carbendazim, is relatively stable in water. It is known that the compound is intact at least 8 days at pH 8 in water.[5,7] Due to these behaviors, the direct measurement of benomyl concentration is not feasible, but indirect method, determination of carbendazim concentration, is widely accepted. The concentration of carbendazim is transformed to benomyl concentration by multiplication of molecular ratio of benomyl to carbendazim.[2,8]

Sample preparation for water sample is straightforward. Benomyl and its metabolite, carbendazim, were extracted by liquid/liquid extraction after hydrolysis of benomyl to carbendazim to ensure complete break down. The final samples containing carbendazim were either analyzed by HPLC/UVD directly or proceed to N-methylation step for GC analysis.[9,10] On the other hand, sample preparation for soil or biota is rather ambiguous. Although generally accepted preparation procedure for soil and biota samples has not been

established so far the samples can be extracted with organic solvent then hydrolyzed under acidic condition similar to sample preparation for water.[10]

Although HPLC/UVD is widely used for the analysis, it has sensitivity problem and possibly incurs positive error in quantitative analysis by the organic interferences those having similar retention time to that of carbendazim. Regardless its high sensitivity, GC/MS analysis technique not only include complicate N-methylation step already, but the yield of N-methylation reaction of carbamate is not clearly understood from a chemistry viewpoint. Our group applied LC/MS (TOF) for the benomyl analysis to improve sensitivity without N-methylation for GC, and to eliminate the error induced by interference materials in the samples.[11] Employing this procedure, our group took the benomyl analysis of water, sediment, and biota to address the fate of the compound.

Experimental Section

Sampling. Water samples were obtained from 28 sites of 5 major rivers or streams in Korea from 30, July to 6, October 1999. Sediment samples were obtained from 10 sites out of 28 sites selected for water samples from 14, October to 22, October 1999. Fish and amphibia were collected for biota sample from nationwide sites in Korea from August 1999 to February 2000. Only flesh of carp and minnow that were separately collected for fish was used for the analysis. Several kinds of frogs were collected separately for amphibia and only leg muscle was used.

Instruments and reagents. Benomyl analysis was performed by using HPLC (Varian 9012Q), mass analysis detector (Micromass LCT), and UV detector (Varian 9050 UVD). Pesticide grade dichloromethane, methanol, acetonitrile, and acetone were purchased and used without purification. Standard reagents, 99% benomyl and carbendazim, were purchased from Aldrich Co. and used directly. The grade of all other reagents and nitrogen gas for blow drying were at least E.P grade and 99.999% purity, respectively. Diazomethane for N-methylation was freshly made prior to use with Diazald kit provided by Aldrich Co.

Treatment and analysis of water samples. To the 2 L Erlenmeyer flask, were added 1000 mL of sample, 14 mL of (1 + 1) sulfuric acid, and magnetic stirrer then stirred for 20 h at ambient temperature for complete hydrolysis of residual benomyl. The pH of resulting mixture was adjusted to 7-8 by dropwise addition of 10 N sodium hydroxide solution. The solution was transferred to 2 L separatory funnel and added 80 mL of dichloromethane. After 2 min's shake, the layers were settled down for 10 min and the organic layer was separated. Repeat the extraction 4 more times, and the combined organic layer was dried over anhydrous sodium sulfate. The dichloromethane solution was decanted to Kunderna-Danish (K-D) concentrator and concentrated to 1 mL. Finally concentrated sample was analyzed by LC/MS (TOF) with 300×3.9 μ bondapak (10 μ) column with 0.8 mL/min elution of methanol and water mixture (1 : 1, v/v). Incoming amount to MS detector was adjusted to 1/12 of eluate and the analyte was ionized by electrospray technique. MH^+ ion of 192.19 was detected by selected ion monitoring (SIM) and used for quantitative analysis.

Treatment and analysis of sediment samples. To the 20 g of homogenized sample, 50 mL of methanol was added and treated sequentially with 15 min's shake, 15 min's ultrasoni-cation, and centrifuge at 3,000 rpm. After decant the upper methanol layer the residue was extracted again by above procedure. Combined methanol solution was concentrated to approximately 1 mL by rotary evaporator or K-D concentrator. The concentrate was transferred to the 50 mL Erlenmeyer flask containing 20 mL of 0.3 N sulfuric acid. After 2 h's stirring at room temperature, adjust the pH to 7-8 by dropwise addition of 10 N sodium hydroxide. The resulting mixture was extracted with 10 mL of dichloromethane as mentioned on water sample treatment (section 2.3). Repeat the extraction twice, and the combined dichloromethane solution was dried over anhydrous sodium sulfate, concentrated by K-D concentrator, and then analyzed by LC/MS (TOF).

Treatment and analysis of biota samples. To the 5 g of homogenized sample, 20 mL of acetonitrile was added. The mixture was shaken for 10 min and filtered through GF/C filter paper. Repeat the extraction twice and the combined filtrate was evaporated to 5-10 mL by rotary evaporator. The concentrate was passed through silica gel cartridge. The flask was washed with 10 mL of methanol and the washed methanol was also passed through the silica gel cartridge and combined. The methanol solution was concentrated to 1 mL approximately, and then 10 mL of 0.3 N sulfuric acid was added to the solution. After 2 hours stirring at room temperature, pH of the mixture was adjusted to 7-8. The mixture and 5 mL of dichloromethane were transferred to separatory funnel and the mixture was shaken for 2 min vigorously then organic layer was separated. After two more extraction, the combined dichloromethane solution was dried over anhydrous sodium sulfate. The dichloromethane solution was decanted and dried under reduced pressure. The residue was dissolved with 2 mL of methanol and directly passed the solution through silica gel cartridge then followed by methanol wash, if the solution has suspended solid. The filtrate was diluted to 5 mL by addition of extra methanol and analyzed by LC/MS (TOF).

Results and Discussion

U.S. EPA-631 method reported the method detection limit (MDL) for benomyl analysis is 8.7 μg/L for water sample but still no MDL data for sediment and biota has been reported.[6] According to the Japanese Speed 98, MDLs for water, soil, and biota samples are 0.1 μg/L, 2 μg/kg, and 20 μg/kg, respectively.[7] Our sample treatment for benomyl analysis in water was carried out according to U.S. EPA-631 method except concentration ratio. According to U.S. EPA-631 method, 500 mL of water was taken and concentrated to final volume of 10 mL. However, our group used 1 L of

sample and concentrated to 1 mL to improve MDL. Japanese Speed 98 method was applied for the treatment of sediment and biota samples with a few modifications. The concentrations of carbendazim in the finally concentrated samples were determined by LC/MS (TOF) with detection of MH^+ ion (m/z = 192.19). Benomyl concentration was calculated by multiplication of 1.52 (molecular weight ratio of benomyl to carbendazim) to the carbendazim concentration, which is directly acquired by sample analysis. Calibration curve was obtained by measurement of blank, 50, 300, 500, and 1000 μg/L standard carbendazim solution. Calibration curve was freshly made just before the actual samples were measured. The correlation value (linearity) was satisfactory with over 0.9962. Stability of the instrument and calibration curve was regularly checked by analysis of 50 μg/L standard carbendazim solution at every 10 samples. Warning and control level were set to ± 2s (standard deviation) and ± 3s, respectively. Recalibration of standard curve was transacted upon over of control level or consecutive over of warning level twice.

MDL was calculated by multiplication of 3.14 (Student T value at 98% confidence level) to standard deviation. In turn, standard deviation was obtained by determination of carbendazim concentrations in 7 samples those are previously spiked to be 0.0304 μg/L and 0.530 μg/kg with standard carbendazim solution for water and soil (include sediment) samples, respectively. As shown on Table 1, MDLs for water and soil determined by LC/MS (TOF) were 0.0053 μg/L and 0.184 μg/kg, respectively. These values are at least 10 times lower than those of reported values on Japanese Speed 98, which are recognized as lowest MDL values so far. Data quality was assured by measuring matrix spike recoveries. Approximately 10% samples were randomly taken and spiked with standard carbendazim solution. Spike recoveries were fairly good with a range of 80-118% and 63-75% for water and sediment, respectively (Table 2).

N-methylation of carbendazim for GC/MS analysis was performed to investigate the recovery yield of Me-carbendazim. The presumed Me-Carbendazim solution, which is made from a mixture of carbendazim and diazomethane, was analyzed by GC/MS to provide the evidence of Me-carbendazim formation. However, the GC/MS chromatogram showed only trace amount of Me-carbendazim and a lot of unidentifiable peaks. These unidentifiable peaks are speculated to be originated from either N-methylation process or thermal decomposition in the GC column. Since the amount of Me-carbendazim detected by GC/MS assumed to be less than 1%, the determination of carbendazim concentration by GC/MS would not be a suitable technique.

Table 2. Spike recoveries of carbendazim in water and sediment samples

River	Spike recoveries (%)	
	Water	Sediment
Han	81.2	74.83
Nakdong	N/A	63.18
Youngsan	80.5	N/A
Sapkyo	N/A	N/A
Samchuck Osip	118.5	N/A

Determination of carbendazim concentration employing LC/UVD provided broadband around the expected retention time of the compound. As seen on Figure 2, chromatograms of actual water sample (one of Youngsan river site) obtained by LC/UVD and LC/MS (TOF) clearly showed the differences. The LC/UVD chromatogram has not only unstable base line but also unsymmetrical carbendazim peak while LC/MS (TOF) chromatogram has clean and symmetrical peak. This phenomena might be occurred due to the superimpose of interfering organic impurities that have the similar retention time to carbendazim. Four samples out of 28 water samples were randomly taken and the carbendazim concentration was determined by LC/UVD and LC/MS (TOF) to verify the assumption. Four samples out of 10 sediment samples were also taken for the test. As shown on Table 3, the carbendazim concentration determined by LC/UVD was turned out to be up to 3.5 times greater than that of de- termined by LC/MS (TOF). The range of the difference between the concentrations obtained by LC/UVD and LC/MS (TOF) was from 0.03 to 0.4 μg/L and totally independent to the concentration of carbendazim. The concentrations of carbendazim in soil samples were also taken by LC/UVD and LC/MS (TOF). The differences of carbendazim concentration determined by the above two detectors were much greater than that of water samples. LC/UVD showed the

Table 1. MDLs for water and sediment (soil) samples

Sample number	Water		Sediment	
	Carbendazim concentration (μg/L)	Benomyl concentration (μg/L)	Carbendazim concentration (μg/kg)	Benomyl concentration (μg/kg)
MDL1	0.026	0.039	0.325	0.494
MDL2	0.024	0.036	0.380	0.577
MDL3	0.022	0.0334	0.426	0.648
MDL4	0.023	0.036	0.435	0.661
MDL5	0.024	0.036	0.384	0.584
MDL6	0.024	0.036	0.359	0.545
MDL7	0.024	0.036	0.363	0.552
Standard Deviation	0.0011	0.0017	0.0385	0.0586
MDL	0.0035	0.0053	0.121	0.184

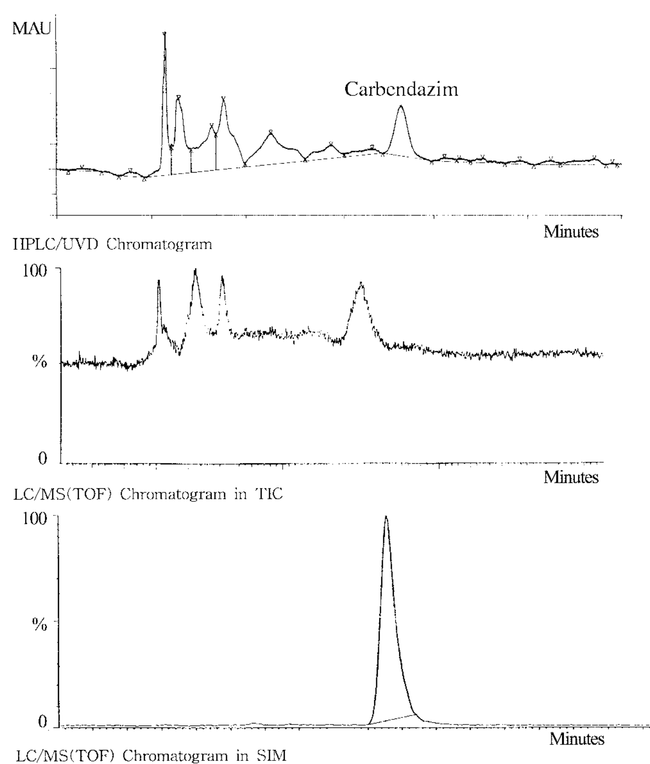

Figure 2. Carbendazim chromatograms of an actual sample collected at Youngsan river site.

carbendazim concentration up to 10 times greater than that of LC/MS (TOF).

These experimental results led us to the following conclusions. The major contribution for the peak broadening of the chromatogram is presumably due to the organic impurities those have the similar retention time to that of carbendazim. Sediment contains more organic impurities and it induced greater deviation than water.

Benomyl concentrations in water and sediment determined by LC/MS (TOF) were shown on Table 4. The range of averaged benomyl concentration value for each river was from 0.070 to 0.586. Ten out of 28 sites for water sample collection are selected for sediment sample collection to overlook the fate of benomyl. The benomyl concentration in soil sample, converted from carbendazim concentration by multiplication of 1.52, was ranged from 0.214 to 5.543 μg/kg. Most of sediment samples were more contaminated than water at least 50 to the maximum of 500 times. Biota was turn out to be free from residual carbendazim contamination regardless fish or amphibia.

These experimental data inferred the assumption, that carbendazim can be accumulated in sediment and the degradation rate in sediment is slower than that in water. The carbendazim, intaken by biota, is so rapidly degraded in vivo that no residual compound was detected.

Since benomyl has two chemically active functional groups (carbamate and urea sites), benomyl can be broken down by the attack of nucleophiles. Although the breaking down pathway can be altered by pH and the nature of the nucleophile itself, the breaking down rate is inevitably increased by the increase of nucleophile concentration and temperature. Carbendazim, adsorbed on the sediment particles, can be not only protected from the attack of nucleophile but also its degradation condition is unfavorable since the environment has lack of light and heat. On the other hand, the degradation rate of carbendazim in biota is increased not only by the abundantly exiting nucleophiles in biota, but also by the biological thermal energy as well. Consequently, no sign of residual carbendazim was detected in biota. Since biological damage is incurred by the simple intaking of carbendazim into the organisms rather than accumulation, no detection of

Table 3. The benomyl concentration in water and soil samples determined by LC/MS (TOF) and LC/UVD.

Sample		LC/MS-TOF (μg/L, µg/kg)	LC/UVD (μg/L, μg/kg)	% differences
Water	W-1 (Nakdong)	0.232	0.372	59.3
	W-2 (Nakdong)	0.059	0.156	160.4
	W-3 (Nakdong)	0.143	0.772	20.3
	W-4 (Youngsan)	0.119	0.536	350.3
Sediment	S-1 (Han)	3.376	6.374	67.2
	S-2 (Nakdong)	1.188	10.887	816
	S-3 (Nakdong)	1.724	3.819	122
	S-4 (Sapkyo)	5.543	54.654	986

Table 4. The benomyl concentration in water and sediment

River	Benomyl			
	Water (μg/L)		Sediment (μg/kg)	
	Number of sampling site	Concentration (Average)	Number of sampling site	Concentration (Average)
Han	10	ND~2.829 (0.586)	3	ND~0.390 (0.214)
Nakdong	10	ND~0.440 (0.111)	4	1.344~1.727(1.180)
Youngsan	6	ND~0.962 (0.195)	1	1.465
Sapkyo	1	0.195	1	5.543
Samchuck Osip	1	0.070	1	1.718

residual carbendazim in the biota does not mean the safe of living organisms from hazardous effect of carbendazim.

Conclusions

Application of LC/MS (TOF) for the determination of benomyl metabolite (carbendazim) turned out to be very efficient. Its MDL was ay least 10 times lower than that of Japanese Speed 98 method for water sample. LC/MS (TOF) also provides more accurate carbendazim concentration data than those of LC/UVD since LC/MS is able to eliminate superimposing signals induced by organic impurities.

Determination of benomyl concentration employing LC/MS (TOF) revealed the wide contamination of water and sediment. No sign of residual benomyl metabolite (carbendazim) in biota was detected. High concentration of carbendazim in soil compare to water was probably due to the accumulation of the compound induced by slow degradation rate of the compound in sediment. No carbendazim accumulation in the biota might be explained by fast degradation of carbendazin in the biota, presumably. Since the factor of testicular lesion occurred by benomyl is not accumulation but direct intake, the environmental contamination of benomyl could give a biological damage to organisms although the compound has no tendency of accumulation in biota.

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