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Extraction and Analysis of Chlordimeform and Demethylchlordimeform from Human Tissue Samples

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Chlordimeform (CDM), an acaricide/insecticide, and its major metabolite, demethylchlordimeform (DMCDM), are members of a chemical class called the formamidines. CDM has been used successfully to control mites on plants and ticks on animals.

The metabolism of CDM and DMCDM in plants and animals has been the subject of numerous investigations, including those of Sen Gupta and Knowles (1969), Knowles (1970), Knowles and Sen Gupta (1970), Benezet and Knowles (1976), and Knowles and Benezet (1977). Radiolabeled compounds were used in all of those studies, and the concentration of the parent compound and/or one of its metabolites was measured in the urine and/or feces by the amount of radiation emitted. Thin-layer chromatography was used in most instances to separate the parent compound from its metabolites.

Other studies have dealt more directly with procedures used in extracting and analyzing the parent compound and/or metabolite(s) from plant and/or animal material. Methodologies outlined in publications by Geissbuhler et al. (1971) and Kossmann et al. (1971) deal with extractions from plant and soil material and includes the use of thin-layer chromatography, colorimetry, and gas chromatography (GC) in separating and quantitating the compound(s). Voss et al. (1973) published an extensive article dealing with formulation and residue analysis of CDM and noted that colorimetry or GC can be used to measure CDM. More recent papers, including those of Witkonton and Ercegovich (1972), Iizuka and Masuda (1979), and Machin and Dingle (1977), contain information relating to the extraction and determination of formamidine compounds from fruit plants, rice plants and paddy soil, and cattle dipping baths and sprays, respectively.

This study was undertaken to investigate the extraction and analysis of CDM and DMCDM from human tissue samples, since the presence of CDM in the environment may lead to acute intoxication in humans. Human whole blood and liver homogenate were used in an attempt to design a relatively simple, yet rapid and accurate method of assaying these two compounds in human tissue samples.

MATERIALS AND METHODS

CDM HCl was supplied by Ciba-Geigy. DMCDM HCl was obtained from the medicinal chemistry department of Purdue University. N'-(2,4-xylyl)-N-methylformamidine HCl (U-40481A) was supplied by Upjohn Pharmaceutical Company. Phenmetrazine HCl was obtained from Boehringer-Engelheim. Working standard solutions of these compounds were prepared from stock solutions by diluting them with 100 percent ethanol to make concentrations of 1 mg/ml. Reagent grade concentrated hydrochloric acid and sodium sulfate were obtained from J.T. Baker Chemical Company, as were chromatographic grade n-butyl chloride, acetonitrile, iso-amyl alcohol, and chloroform. The deionized water used throughout the study was prepared from mixed resin beds purchased from a local distributor.

A Hewlett-Packard 5840 GC equipped with a flame ionization detector (FID) and a 1.8 m column packed with 3% OV-17 on 100/200 mesh Gas-Chrom Q was used in the first part of this study. A Hewlett-Packard 5985-A Gas Chromatograph/Mass Spectrometer (GC/MS) equipped with electron impact ionization and a 1.5 m column packed with the same 3% OV-17 on 100/200 Gas-Chrom Q material was used in the latter part of the study.

The basic approach in developing a simple, yet accurate and rapid method of extraction and analysis for CDM and DMCDM was to extract them from tissue samples with an organic solvent, transfer them to an acid aqueous medium, and then back extract into a relatively small volume of an organic solvent before injecting an aliquot into a GC or GC/MS. The choices of best organic solvent and optimal volume and pH of the acid aqueous medium to be used were evaluated. CDM (10 ug samples) was extracted from deionized water using four different organic solvents or mixtures thereof: 1) chloroform, 2) n-butyl chloride, 3) a mixture of chloroform and acetonitrile (20:1) and 4) a mixture of chloroform and amyl alcohol (10:1). The samples were extracted with 100 ml of solvent in a 125-ml separatory funnel. Five different concentrations of dilute hydrochloric acid (0.1, 0.2, 1.0, 2.0, and 6.0 N) were tested for optimal acidity of the aqueous medium. Additional tests on optimal volumes of dilute hydrochloric acid to use was evaluated using 5 and 10 ml portions.

An extraction recovery study was carried out following the development of a step by step extraction procedure. CDM and DMCDM (1, 5, and 25 ug of each) were extracted from three different types of media: deionized water, human whole blood, and human liver (1:1 aqueous homogenate). U-40481A was used as an internal standard and added in equal amounts. An unextracted control containing CDM, DMCDM, and U-40481A in equal amounts (5 ug on the column) was used. The peak area ratios of CDM and DMCDM to U-40481A from extracted samples was compared to the peak area ratios

of the unextracted sample. A minimum of four extractions per sample size and three analyses per extract were made in the recovery study.

Two concentration ranges of CDM and DMCDM were studied (1, 3, and 5 ug samples in the first series, and 15, 20, 25, and 30 ug samples in the second) to determine whether CDM and DMCDM would extract in a linear fashion using the extraction procedure developed. U-40481A was used as an external standard in quantities of 3 and 20 ug, respectively, for the two series. Each sample was extracted three times and each extract was analyzed three times.

RESULTS AND DISCUSSION

Of the four organic solvents or mixtures thereof evaluated in this study, chloroform and n-butyl chloride were shown to be the most suitable. Table 1 shows that recoveries of 81 and 75 percent were obtained with chloroform and n-butyl chloride, respectively, and much less with the other solvent mixtures. Table 2 shows the percent recovery of CDM when acidic solutions of various strengths (normality) and volumes of 5 and 10 ml were used in the extraction. Recoveries were highest (83.6%) when 10 ml of 0.1 N HCl was used. Ten ug of phenmetrazine was used as an external standard in this part of the study. Two ul of each extract was injected three times into a Hewlett-Packard 5840 GC with a FID and the mean peak area ratios of unextracted controls to extracted samples were compared to determine the percent recovery. Figure 1 shows a typical chromatogram of CDM and phenmetrazine.

Table 1. Percent Recoveries of 10 ug of CDM HCl Dissolved in Deionzed Water. Values are means ± S.D. N = 3.

Extraction Solvent	Percent Recovery
Chloroform	81.0 ± 0.8
n-Butyl chloride	75.0 ± 0.3
Chloroform : acetonitrile (20 : 1)	52.6 ± 0.7
Chloroform : isoamyl alcohol (10 : 1)	38.3 ± 0.3

Table 2. Percent Recoveries of 10 ug of CDM HCl Dissolved in Deionized Water. Values are means ± S.D. N = 3.

Normality and Volume of HCl	Percent Recovery
0.1 Normal; 5 ml	75.7 ± 0.7
0.1 Normal; 10 ml	83.6 ± 0.4
0.2 Normal; 5 ml	72.7 ± 0.5
1.0 Normal; 5 ml	34.6 ± 0.5
2.0 Normal; 5 ml	11.0 ± 0.2
6.0 Normal; 5 ml	7.0 ± 0.4

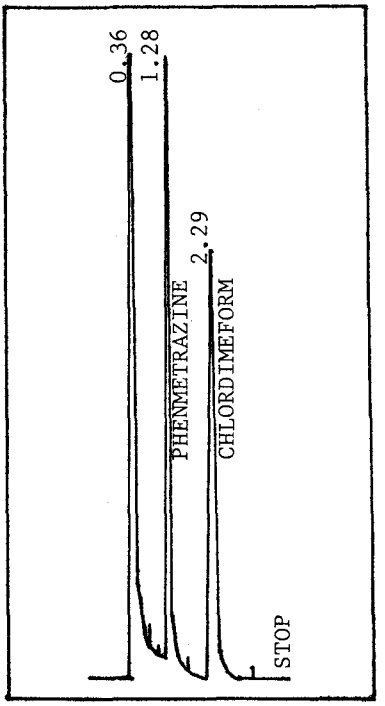

Figure 1. Gas chromatogram of phenmetrazine and CDM obtained with a Hewlett-Packard GC 5840 equipped with a FID and a 1.8 m column packed with 3% OV-17 on 100/200 mesh Gas-Chrom Q and a carrier gas flow of 30 ml/min of N_2; Injector and detector temperatures were 270° C.

Chlordimeform, as the free base, is highly soluble in organic solvents and only slightly soluble in water (250 ppm), whereas CDM HCl is very soluble in water (more than 50%) and only slightly soluble in organic solvents (Voss et al. 1973). Concentrated NH_4OH was used to convert CDM to free base, thereby facilitating its partitioning into the organic medium at the beginning of the extraction and its transfer out of the acid, aqueous phase into the relatively small volume of chloroform near the end of the extraction procedure. CDM free base is a liquid at room temperature and is quite volatile (3.5×10^{-4} Torr at 20° C). During the initial stages of this study, recoveries were occasionally unexpectedly low. The use of an ice bath near the end of the extraction (step 9 below), when the compound was transferred from the acid aqueous phase into a small volume (200 ul) of chloroform, solved the problem and ensured consistently good recoveries.

STEPWISE PROCEDURE FOR EXTRACTION OF CDM AND DMCDM:

1. Place substance to be extracted in 125-ml separatory funnel; add necessary amount of internal standard.

2. Add 5 drops of concentrated ammonium hydroxide and swirl slightly.

3. Add 100 ml of chloroform, stopper the separatory funnel, and extract by shaking for 5 min.

4. Let the phases separate and filter off the lower chloroform layer through a Whatman 541 filter paper containing approximately 1 g of sodium sulfate into another 125-ml separatory funnel.

5. Add approximately 10 ml deionized water to the separatory funnel and extract by shaking for 5 min.

6. Repeat step 4.

7. Add 10 ml of 0.1 N HCl to the separatory funnel and extract by shaking for 5 min.

8. Let the phases separate and discard the lower chloroform phase.

9. Place the aqueous 0.1 N HCl phase into a 15-ml conical centrifuge tube and then place the tube and its contents in an ice bath.

10. Add 1 ml of concentrated ammonium hydroxide and 200 ul of chloroform to the contents of the tube.

11. Cap the tube and vortex mix for 5 min.

12. Centrifuge the contents of the tube for 5 min at 3000 rpm.

13. Aspirate off the upper aqueous phase leaving a small amount to keep the chloroform from evaporating.

14. Withdraw an appropriate amount of the chloroform layer containing the compound and/or internal standard and inject it into the analytical instrument.

The extraction recovery study was performed using the stepwise procedure outlined above and a Hewlett-Packard 5985-A GC/MS to quantify the compounds. This instrument's computer capabilities permitted the monitoring of single ions and integration of peak areas. A typical chromatogram is shown in Figure 2. The least amount of CDM that could be detected with this instrument was

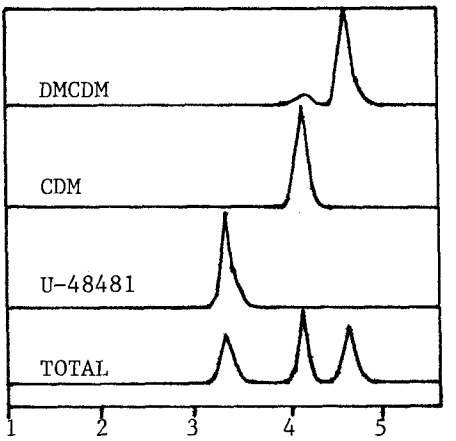

Figure 2. Hewlett-Packard 5985-A GC/MS record. Three single-ion chromatograms and one total-ion chrmatogram are shown. Injector temperature = 270°C. Column temperature programmed at 150-260°C, @ 10° C/min. Carrier gas flow = 35 ml/min of helium.

0.05 ug. Aliquots of 1 µl were injected to minimize potential adverse effects of the halogenated solvent on the detector. The use of GC-FID would circumvent this problem.

Table 3 shows that close to 84 percent of CDM and 72 percent of DMCDM could be recovered from deionized water. Recoveries were about 8 to 12 percent less from human whole blood and about 20 percent less when extractions were made from human liver homogenates. Recovery of DMCDM was consistently 10 or so percent less than that of CDM from any given medium or sample size. This is likely due to slight differences in physicochemical properties between the two compounds. The size of sample used (1, 5, or 25 ug) made no difference in percent recovered ($P \leq 0.05$) as judged by an analysis of variance, regardless of which compound or which medium was used.

Table 3. Percent Recoveries of CDM and DMCDM from Three Different Types of Media: Deionized Water (DW), Human Whole Blood (HWB), Human Liver Homogenate (HLH), Values are Means ± S.D. N = 4.

Extraction Media	1.0 ug	5.0 ug	25.0 ug
CDM, DW	82.0 ± 2.5	83.9 ± 1.1	85.1 ± 1.2
DMCDM, DW	67.2 ± 1.6	71.1 ± 2.4	77.2 ± 2.1
CDM, HWB	71.1 ± 1.7	69.2 ± 1.6	73.5 ± 2.7
DMCDM, HWB	62.8 ± 2.2	66.4 ± 2.8	61.5 ± 3.1
CDM, HLH	64.9 ± 0.5	63.8 ± 1.7	62.9 ± 2.6
DMCDM, HLH	56.9 ± 0.1	48.0 ± 0.1	52.5 ± 2.1

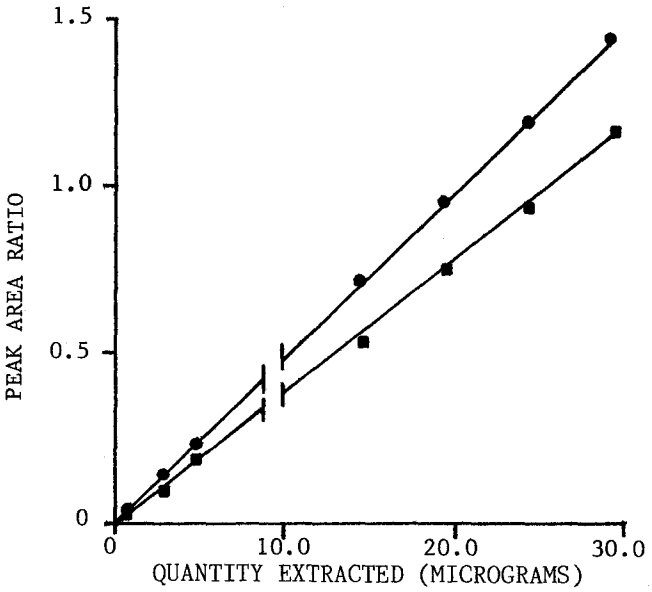

Figure 3. Extraction Linearity for CDM and DMCDM. Linearity determined by least squares regression analysis.

Figure 3 shows that CDM and DMCDM extractions are independent of amount present in the range studied. Data obtained in the extraction studies were obtained with the Hewlett-Packard GC/MS, but good results could be obtained with a GC equipped with a flame ionization detector.

One of the most widely used methods of extraction and quantification of CDM and its metabolites (including DMCDM) is that of Kossmann et al. (1971). Their method is more lengthy than the present one and involves two initial extractions (methanol-HCl and methanol-dichloromethane), evaporation of the solvents, and three partitioning steps. Their recovery rates from CDM ranged from a low of 72 (1.0 ppm added to grapes) to a high of 119 percent (0.8 ppm added to prunes). Recovery of DMCDM ranged between 70 (0.5 ppm added to pears) to 91 percent (0.8 ppm added to prunes). A colorimetric or GC procedure was described for quantification. Voss et al. (1973) reported good recoveries of total CDM residues when it was added to several types of fruit (apples, peaches, pears, and plums), eggs, chicken and bovine meat, and bovine liver, fat and milk. Their percent recoveries ranged from a low of 88 (bovine fat) to a high of 105 percent (apples). Their extraction procedure was quite complex compared to the present one. It involved hydrolysis and steam distillation and required the use of a distillation-extraction head, which is not commonly found or used in most laboratories. The diazo moiety of CDM and other derivatives had to be exchanged for iodine to obtain a derivative that could be measured with a GC equipped with

an electron capture detector.

Foerster et al. (1978) published an interesting report on the extraction and analysis of over 40 basic drugs from blood by using a relatively simple procedure of solvent extraction, partitioning with an acid aqueous phase, and quantification with GC. Their recoveries ranged from 27 for methyprylon to 103 percent for methapyrilene, although most were in the 80 to 90 percent range. Their procedure, like ours, is relatively simple, rapid, and devoid of any need for costly or unusual equipment. Both have limitations, but appear adequate for use as an initial screen for detecting acute intoxication in humans.

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