General Protocol for the Acute Time Course and Dose-Response Studies of the Individual Carbamate Pesticides in Adult Male Rats

Carbaryl, carbofuran, methomyl, oxamyl, methiocarb, formetanate-HCL, and propoxur are seven carbamate pesticides used to control pests in the United States. All seven of these pesticides are cholinesterase inhibiting pesticides and act by inhibiting acetylcholinesterase, the enzyme that breaks down the neurotransmitter acetylcholine. The rodent, oral LD_{50}s for these pesticides are listed below:

Carbamate Pesticide	Rat Oral LD_{50} (taken from MSDSs)
Methomyl	14-20 mg/kg
Methiocarb	10 mg/kg
Oxamyl	2.5 mg/kg
Carbaryl	230 mg/kg
Carbofuran	5-7 mg/kg
Formetanate HCL	21 mg/kg
Propoxur	69 mg/kg

Purpose

The overall purpose of these studies is to determine if the interaction of these seven pesticides in a mixture produces a dose-additive interaction (Berenbaum, 1985;Wessinger, 1986).

Test Substances

The test substances are listed above in the Background section. These compounds are cholinesterase inhibitors, and their systemic toxicity is though to be related to that mechanism of toxicity. Five of these compounds, methomyl, methiocarb, carbofuran, formetanate and oxamyl, have LD_{50}s below 50 mg/kg and therefore require an approved safety protocol.

All chemicals will be ordered from Chem Service, Inc., West Chester, PA (610-696-3026) at the highest purity available. Appropriate vehicles will be determined for each pesticide, but it is anticipated that the vehicle will be either corn oil or water. If the chemical is particularly resistant, a small amount of acetone may be used to dissolve the pesticide and then corn oil will be added.

Carbamate Pesticide	Molecular Weight	Chem. Service Catalog #s
Methomyl	162.23	PS-775
Methiocarb	225.33	PS-543
Oxamyl	219.29	PS-737
Carbaryl	201.24	PS-84
Carbofuran	221.3	PS-754
Formetanate	257.75	PS-400
Propoxur	209.27	PS-551

Methods/Experimental Approach:

All animals used in these studies will be adult (90 days old), Long-Evans male rats obtained from Charles River. Rats will be weight-ranked and assigned to groups the day prior to the dosing. Stainless steel feeding needles (18x1-1/2" w/2-1/4mm ball) & Glas-Pak 1 cc syringes will be used for all dosing. Rats will be gavaged at 1 mg/kg body wt. for all studies. Rats will be individually housed in polycarbonate cages (8x8x8) bedded with pine shavings. They are given food (Purina Rat Chow 5001) & water (filtered tap water, automatic watering system) *ad lib.* The holding room is on a 12 hr light: 12 hr dark cycle.

The studies will be the following:

1. The first study with the individual compounds will consist of determining the time of peak effect (defined as brain cholinesterase inhibition) and the approximate time of cessation of that effect. To perform that study, adult male animals will be obtained from Charles River (90 days old, will be allowed to acclimate for a week, and then be treated). The animals will receive a non-lethal, but effective (to be determined from the literature and also from data obtained from the Office of Pesticide Programs) oral dose of the pesticide in an appropriate vehicle (corn oil, water, or acetone/corn oil), and will be observed periodically for up to 8 hrs on the day of dosing, and at 24 hours, for overt cholinergic toxicity (*e.g.*, salivation, lacrimation, tremors). After a behavioral time of peak effect has been estimated, another, larger group of animals will be dosed with an effective, but non-lethal dose of the same carbamate, and animals killed at appropriate times to be determined from the first pilot study (examples of appropriate times include the time of peak effect, and times surrounding the time of peak effect, and also a time when the signs are no longer apparent). The animal will be anaesthetized with carbon dioxide and then decapitated; the trunk blood and brain will be collected. The blood will be centrifuged for 10 minutes at 1000 x g to separate plasma and red blood cells (RBCs). Plasma will be collected and frozen at -80°C; RBCs will be diluted (1:3; initial to final) with Ellman buffer containing 1% Triton x-100 and frozen at -80°C. Whole brains will be collected from each animal

and stored at -80°C until cholinesterase analysis. The RBCs and brain will later be analyzed for cholinesterase activity using the radiometric method of Johnson and Russell (1975)(NHEERL-NTD-CMTB-SP/SOP-001-001). Samples of the same tissues will also be analyzed by the spectrophotometric method of Ellman (Ellman *et.al*, 1961) using the Hitachi 911 analyzer (NHEERL-NTD-CMTB-SP/SOP-002-000). These assays will take place in parallel (*i.e*., on the same day). This is to compare the data obtained by these two methods. There will be four to five treated animals killed at each time point. In addition, there will be five control animals dosed with vehicle that will be distributed throughout the time points. The vehicle will be corn oil, water, or acetone/corn oil, and the animals will be dosed by gavage, 1 ml/kg.

Data obtained from this study: a time course of RBC and brain cholinesterase activity using two different methods in animals dosed with each of the seven carbamate pesticides.

2. The second group of studies will be a dose-response study of each compound alone. The endpoints will be cholinesterase inhibition in brain and RBCs, toxicity score ranking and motor activity assessment, performed at 40 minutes after dosing. The doses chosen will be designed to define the dose response curve for each pesticide between zero and 50-60% brain cholinesterase inhibition. Animals will be dosed with five levels of non-lethal dosages of each pesticide, tested in the motor activity chambers (see below), and blood and brain tissue will be collected at 40 minutes after dosing. For the biochemical endpoints, it is anticipated that there will be five animals in each group with a total of 6 groups (5 dosed and one control) for a total of 30 animals for each pesticide. For the behavioral endpoints, it is anticipated that there will be ten animals in each group with a total of 6 groups for a total of 60 animals. The animals taken for biochemical analysis will be a subset of the 60 animals used for motor activity testing.

For each compound, rats will be relocated to a holding room adjacent to the maze room following weighing and will be allowed to acclimate for a minimum of 30 minutes. The general procedure will be to dose, perform a toxicity scoring and assess motor activity in squads of 4 animals (counterbalanced across all dosage groups). Twelve minutes after dosing, rats will be assessed for a toxicity score [scored on a scale from 1 (nothing) to 3 (very obvious)] that defines the presence of cholinergic effects by an observer blinded to each rat's treatment level. At 15 minutes post dose, rats will be placed in the Figure-8 motor activity chambers for a 20 minute session. At 40 minutes post dose (*i.e*., up to 5 minutes after removal from the maze), the rats will be sacrificed according to NHEERL-NTD-CMTB-SP/SOP-04-006-001, and brain and whole blood will be collected from each rat. The tissues will be prepared and stored as in the time course experiment above, and the cholinesterase activity will be determined as described above.

Because of the number of animals in these studies, the studies will be performed on two consecutive days, with the dosing solutions being made fresh for each day's dosing.

Data obtained from this study: RBC and brain cholinesterase activity using two different methods, toxicity score for each animal, motor activity for each animal given one of 5 dosage levels (plus vehicle) of seven carbamate pesticides.

Equipment and Supplies:

Equipment necessary to perform this work is as follows & is located in B254A (unless otherwise noted):

Water bath
Analytical balance
Computer-based Rodent Weigh Station
Vortexers
Stopwatch/Timer

Pipettes (various volumes)
Bench top centrifuge
Liquid scintillation counter (located in B255)

Consumable supplies necessary to perform this work are as follows:

Scintillation vials (7 & 20 ml)
Lab glassware (various)
1 cc Dosing syringes
18 gauge stainless steel feeding needles
Corn oil (Sigma)
Triton X-100 (Sigma)
1.5 ml Micro centrifuge tubes
Ice

All equipment & supplies are considered standard laboratory devices or items and do not need to meet critical specifications.

Animal Husbandry:

All rats will be obtained from Charles River, Raleigh, North Carolina and will be individually cages. All animal work has been approved by the IACUC.

Records:

Day to day records will be kept in laboratory notebooks. All records from the scintillation counter and computer files will be kept in a loose-leaf notebook. All data for each carbamate will be kept in loose-leaf notebooks, with one notebook each for each compound. Each notebook will have dividers for each study. All records having to do with this experiment will have the designation: **CM**(for carbamate mixture)-pesticide used, (**MC, MO, OX, CB, CF, FR or PX**)-study (**1 or 2**)- tissue (**RBC, BR**)-(dose or time point) - individual animal ID number.

All records will be stored in the laboratory and on the laboratory computer hardrive, and on the F drive on the network (this should provide a duplicate of everything), and also on the X drive (a shared drive for all of the people working on this study).

Quality Control Requirements:

The balances are checked before operation each day using National Bureau of Standards certified weights. A range of weights is used to bracket the weight of the samples to be determined that day. Acceptance criteria: between 0 and 100 mg = .1 mg; above 100 mg = 1.0 mg. Records are kept in a dedicated laboratory notebook.

The pH meter is calibrated before each operation using standard pH solutions. A pH within 0.5 pH units of the sample is chosen for calibration. Acceptance criterion: pH must be within .05 units. Records are kept in a dedicated laboratory notebook

The balances and pH meter are also checked semiannually for precision by a contractor.

The scintillation counter is checked before each run in two ways. The factory supplied ^{3}H, ^{14}C and blank standards are run and compared to historical controls. Also, a factory supplied ^{3}H quench curve is run to determine the efficiency of counting and also to make sure the quench curve matches historical controls. Acceptance criteria: within 5% of previous values. All the information from the factory supplied ^{3}H, ^{14}C and blank standards are stored automatically

on the scintillation counter. All the information from the quench curve is entered into an Excel spreadsheet (the basic sheet is on the X drive in the *Quality Assurance* folder with the name *scintillation counter calculations.xls*). This file should be filled out according to the directions and a quench curve plotted and the efficiency calculations conducted. The file should then be saved using the extension of the date of analysis. The quench curve is plotted using SigmaPlot and compared to previous curves. All this information is stored in a loose-leaf notebook in the cabinet under the scintillation counter.

The Hitachi 911 is under a maintenance contract and is checked for performance every three months by the manufacturer's service representative. Additionally, laboratory personnel perform certain monthly and daily checks. These procedures are documented in a looseleaf notebook located in the lab close to the instrument. Some maintenance functions are updated on the analyzer's **Maintenance Screen**.

All dosing solutions will be FEDEXed as soon as possible after each experiment is completed to the EPA analytical laboratory in Fort Meade, MD for analysis. Each shipment will consist of the actual dosing solutions and a vehicle control. Records from each shipment (certification of receipt) will be kept in the loose-leaf notebooks in the appropriate section.

Before each cholinesterase analysis (using either method), control standards will be run. These control standards consist of a brain homogenate (in Ellman/Triton buffer) which has undergone serial dilution: 1:50, 1:100, 1:200, and 1:400. These samples were made from the same brain and stored in color coded microfuge tubes in the -80°C Revco freezer in B-254. The relationship between the samples must be within 10% of historical analysis of these same samples. All these data are to be entered and analyzed in an Excel file named *Brain Standards on Radiometric.xls* on the X drive in the *Quality Assurance Folder*. The results of the control standards run on the Hitachi 911, along with the instrument calibration conducted prior to each assay, are filed in the notebook located in the lab. The analyzer will flag any control value that deviates from its control mean by more +/- 6% on the printout with an "H" or "L." The analyzer also keeps a cumulative record for each control of the last 20 runs in the **QC Screen**.

All samples will be run in duplicate. For the Ellman method, if the duplicates are not within 15% of each other, the samples should be rerun (911 analysis) as quickly as possible. In the case of the radiometric analysis, where the results are not known until the next morning, no samples can be rerun and no values will be thrown out.

Because all data from either the 911 analysis or the radiometric analysis must be entered by hand into spreadsheets, each time the raw data is entered, it will be double checked before analysis by an independent observer. This independent observer will double check at least 10% if the entries, and then initial the page showing that they have double checked the data. Once the two analyses show no discrepancies, the data may be plotted for presentation.

The Revco freezer, refrigerator, and freezer compartment of the freezer will be checked at least 5 days each week for proper temperature and recorded in a laboratory notebook for that purpose. The freezer cold room has its own built in controls complete with alarm.

References:

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Ellman, G.L., Courtney, D.K., Andres, V., and Featherstone, R.M. (1961). A new rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol*.

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Johnson, C.D., and Russell, R.L. (1975). A rapid, simple radiometric assay for cholinesterase, suitable for multiple determinations. *Anal. Biochem*. **64**, 229-238.

Wessinger, W.D. (1986). Approaches to the study of drug interactions in behavioral pharmacology. *Biobehavioral Reviews* **10**, 103-113.