Toxicokinetics in the National Toxicology Program

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BACKGROUND

The National Toxicology Program (NTP) was established by the Department of Health and Human Services (DHHS) in 1978 to coordinate and manage the department's toxicology studies. The objective of DHHS' toxicology efforts is to develop scientific information necessary to protect the health of the public from exposure to hazardous chemicals. The NTP consists of the National Institutes of Health's (NIH) National Institute of Environmental Health Sciences (NIEHS), the Centers for Disease Control and Prevention's (CDC) National Institute of Occupational Safety and Health (NIOSH), and the Food and Drug Administration's (FDA) National Center for Toxicological Research (NCTR). The NTP is administered by a director, who is also the director of NIEHS. An executive committee consisting of heads of Federal health research and regulatory agencies (Consumer Products Safety Commission (CPSC), Environmental Protection Agency (EPA), FDA, National Cancer Institute (NCI), NIEHS, NIOSH, and Occupational Safety and Health Administration (OSHA)) and a board of scientific counselors (composed of governmental, industrial, and academic scientists) provide guidance for the program (figure 1).

The NTP toxicological evaluations are initiated on chemicals that are nominated for study. These nominations can come from the private or public sector, and are sent to Chemical Nominations, National Toxicology Program, NIEHS, PO Box 12233, Maildrop A001, Research Triangle Park, NC 27709. After an extensive nomination review process, selections are made and each chemical assigned to an NTP scientist who serves as the toxicology study project director. A project team is then formed by the project director to review the literature and identify data gaps. Basic information on chemical disposition, chemistry, genetic toxicology, and health and safety are developed as needed and provided to the project director for consideration in the design of toxicology protocols. The toxicology study protocols that are developed may include a general toxicological evaluation and/or specific target organ studies (figure 2).

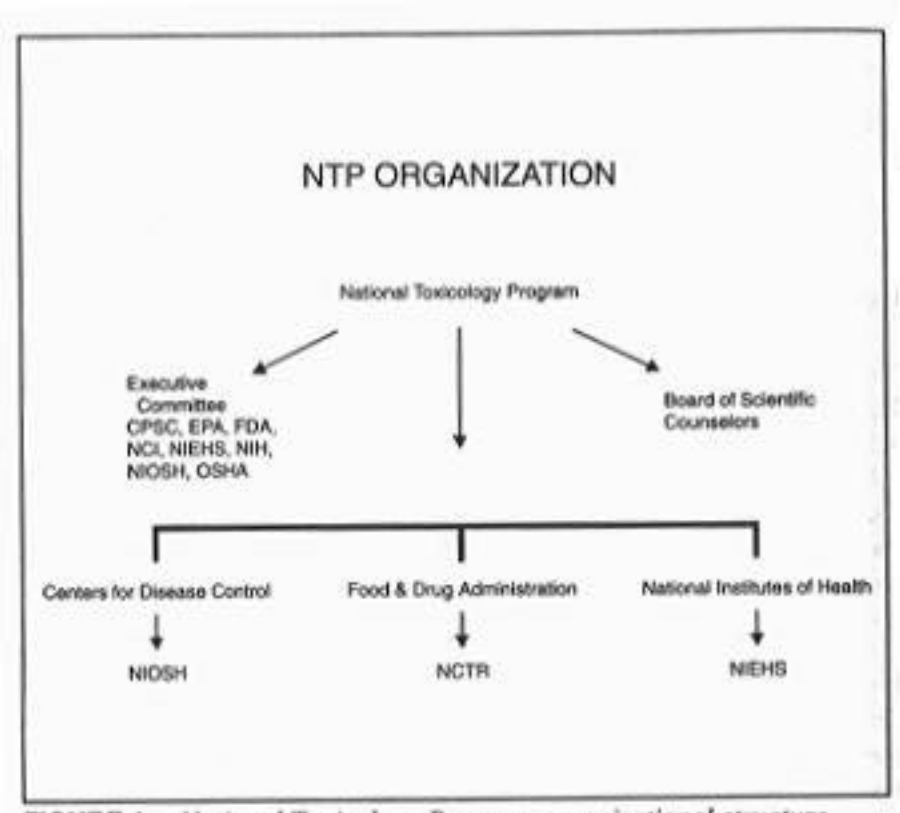

FIGURE 1. *National Toxicology Program organizational structure.*

OVERVIEW

Toxicokinetics is a term used for describing kinetic studies conducted in conjunction with toxicology evaluations (Di Carlo 1982) that deal with absorption, distribution, and elimination processes of chemicals present at concentrations that produce toxic effects. By monitoring the blood concentrations of the chemical and/or metabolites over time after administration by different routes, the test chemical's bioavailability and kinetic characteristics can be readily obtained. The data also permit the determination of the so-called linear dose range based on area under the plasma versus time curve and clearance or other related toxicokinetic parameters, as well as the prediction of possible bioaccumulation after multiple doses. Changes in kinetic parameters after multiple exposures

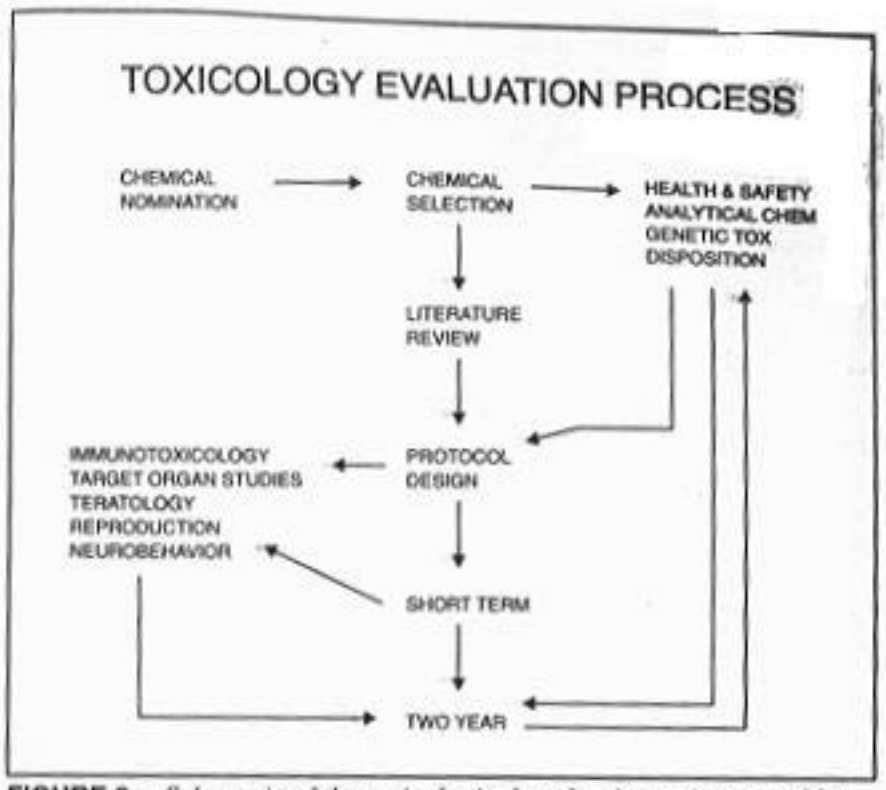

FIGURE 2. *Schematic of the toxicological evaluation process used by the National Toxicology Program.*

are indicative of perturbations in the normal way the animal handles the chemical (e.g., enzyme induction or inhibition). Techniques such as physiologically based toxicokinetic (PBTK) modeling have been used (Bischoff 1967; Ramsey and Andersen 1984; Teorell 1937) to predict the target organ and blood concentrations at various doses. Other work has centered on developing interspecies scaling factors (Mordenti 1986; Travis and White 1988).

During recent years, the importance of toxicokinetic information in risk evaluation has become more widely recognized (Yacobi et al. 1989), including by the NTP. In fact, as part of the activities prior to the conduct of an NTP toxicology study, the determination of basic toxicokinetic information is often conducted to aid in study design. During the conduct of NTP toxicology studies, evaluation of internal dose and changes in kinetic parameters are also frequently conducted to aid in the interpretation of toxicology study outcomes. Generally the internal dose is based on plasma concentrations, since the target organ for the toxicologic effect generally has not been identified as yet. However, after a PBTK model has been developed,

the target organ dose can later be estimated using the collected plasma data. Most of the NTP effort to date has been given to 2-year toxicology and carcinogenicity studies. The application of NTP's toxicokinetic studies to toxicology study design and interpretation is discussed in this chapter.

GOALS

Over the years, NTP, a worldwide leader in toxicology, has conducted chemical disposition studies. More recently, NTP and others (Jameson and Goehl 1994; World Health Organization 1986) have placed even greater emphasis on such studies, especially those that evaluate the kinetics. The scope of the NTP toxicology effort can be seen in the listing of projected NTP toxicology studies for Fiscal Year 1996 (figure 3). Many of the studies listed will include toxicokinetic evaluations. It is anticipated that the toxicokinetic data collected will ultimately improve the usefulness of the toxicology study for risk characterization.

Specifically, the goals of the toxicokinetic evaluations conducted prior to the conduct of toxicology studies are to assist in the selection of the animal species and strain, dose vehicle, dosing route, and dosages. The goals of the determination of concentrations of test chemical or metabolite concentrations in biological samples during the course of the toxicology study are to estimate the internal dose and its possible correlation to toxic effects, as well as to evaluate the effect of age and multiple exposure on the disposition kinetics (figure 4).

APPROACH

If the available toxicokinetic information does not provide enough information to aid in toxicology study design, NTP designs an upfront toxicokinetic study. The doses for the toxicokinetic study are chosen to reflect those anticipated to be used in the toxicology studies. The most common species used by the NTP are Fischer 344/N rats and B6C3F1 mice. The approach used by the NTP involves three steps: preliminary

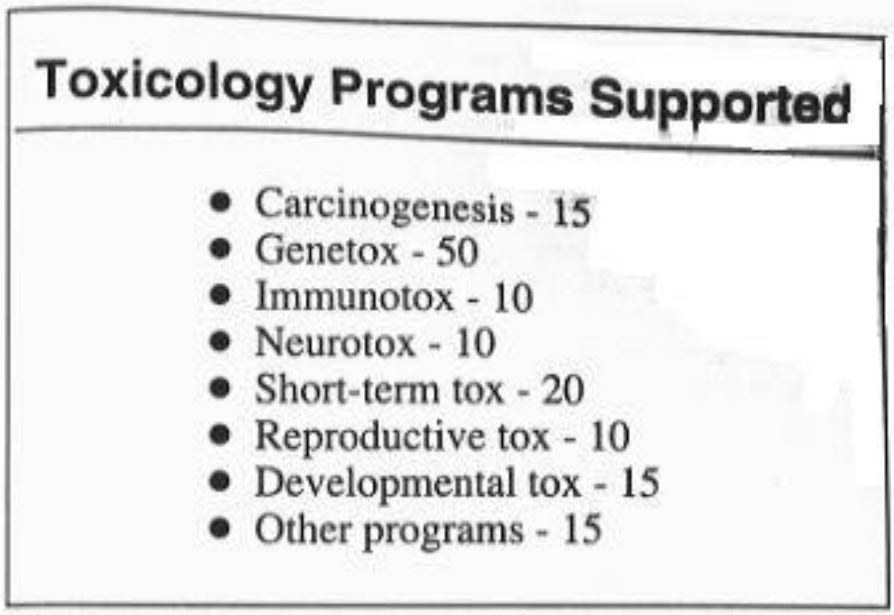

FIGURE 3. *List of the number of chemicals in each program area scheduled for study by the National Toxicology Program in FY 1996.*

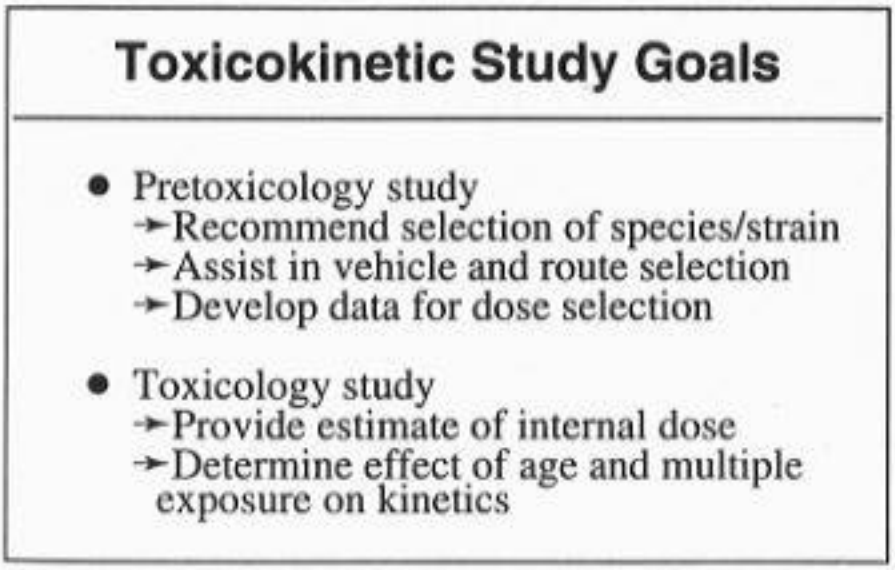

FIGURE 4. *Goals for the conduct of toxicokinetic studies prior to and during the actual toxicology study.*

studies, definitive studies, and studies conducted during the in life toxicology evaluations. The first step in producing the needed toxicokinetic data is to develop an analytical method to quantify the test chemical or metabolite in a biological sample. Most of the time blood plasma is used; all subsequent discussions will specifically refer to the analysis of the analyte in plasma. If the anticipated plasma concentrations and optimal blood sampling times are not known, it is often necessary to conduct a preliminary toxicokinetic study. This type of study involves the administration of the test chemical at the lowest and highest doses proposed for the toxicology study to a small number of animals by appropriate routes and then drawing blood samples at approximately 12 time points. The samples are analyzed using an unvalidated analytical method. Based on these results, the analytical method is further developed to accommodate the determined concentration range and then validated (see next section on analytical method validation). The stability of the analyte in the biological material is subsequently determined at 75 percent of the highest expected concentration for the period of time over which the samples are to be stored prior to analysis (figure 5).

The second step is to conduct definitive toxicokinetic studies (figure 6). In the initial single-exposure definitive study, the chemical is intravenously administered to the animals at two doses, using sufficient animals so that three data points are available at each of 10 sampling times. (The number of sampling times depends on the kinetic profile suggested by the preliminary toxicokinetic studies.) It is hoped that the intravenous (IV) single-exposure definitive study results in plasma concentrations that are directly proportional to dose and provides basic toxicokinetic parameters. The IV study is conducted regardless of the exposure route planned for the toxicology study.

In the case of toxicology studies that will use the gavage or dermal route of exposure, additional animals are dosed by gavage or dermally at three doses covering the proposed toxicology study dose range, again using sufficient animals so that three data points will be available at each sampling time. Often the doses for the toxicokinetic evaluation are the minimally toxic dose (MTD) and fractions of the MTD. This study allows for the determination of the absorption rate, dose proportionality, and, along with the results of the IV route study, the bioavailability. It is important to note that since the interpretation of the results of the toxicokinetic studies depends on the accuracy of animal dosing, the dose

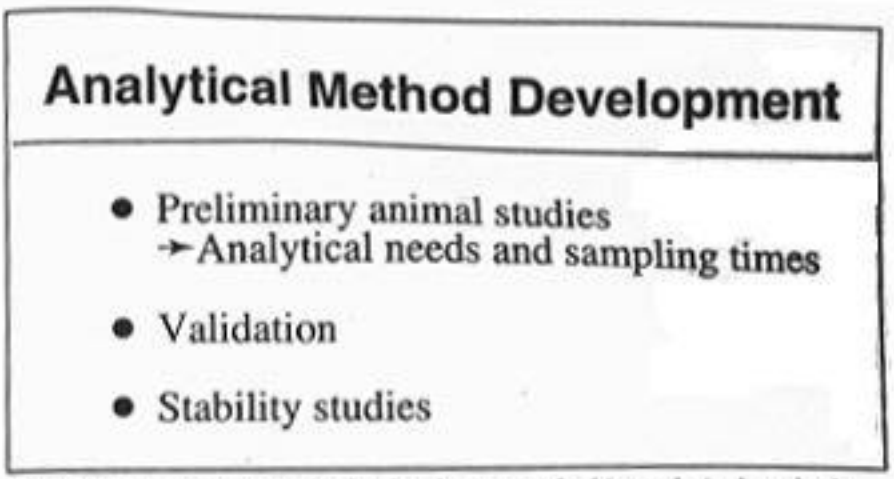

FIGURE 5. *Basic steps in the development of a bioanalytical method.*

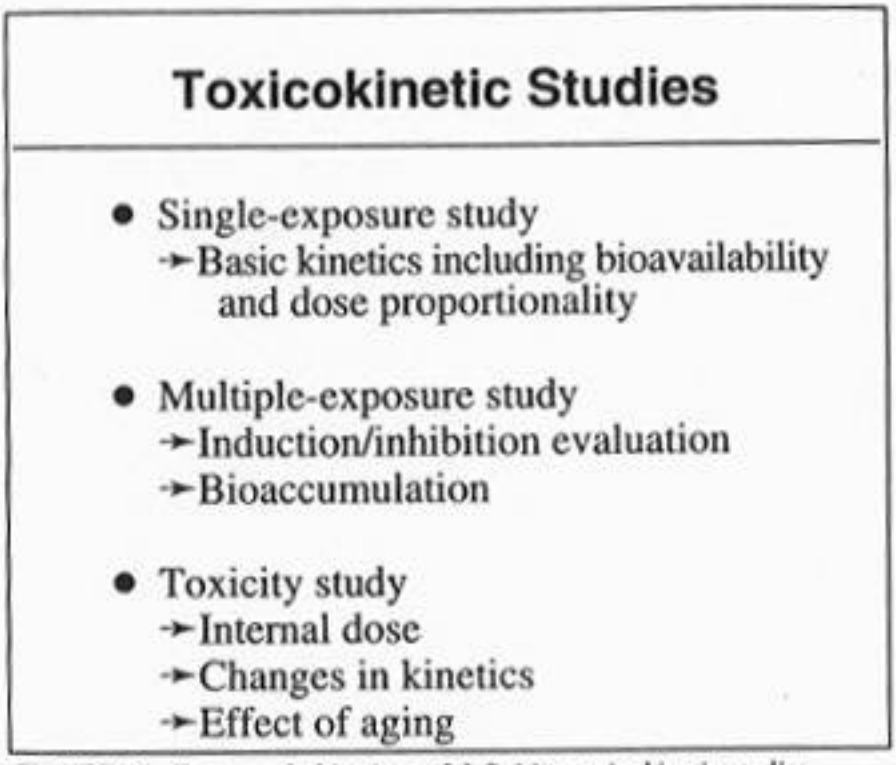

FIGURE 6. *Types and objectives of definitive toxicokinetic studies.*

formulations need to be characterized by homogeneity, stability, and accuracy of dose preparation. A number of the NTP 2-year

toxicology and carcinogenicity studies use the feed or drinking water route for test chemical exposure. How to use the kinetic data from single bolus dose studies in the design and evaluation of feed or drinking water studies is a challenge to the toxicokineticist. To help address this issue, a computer model was developed (Yuan 1993) to incorporate the animal feed or drinking habits together with the kinetic behavior of chemicals to predict the blood concentration profiles during dosed-feed or drinking water studies. The needed absorption rate and dose proportional range are determined from kinetic studies using the gavage route. The top dose for the gavage toxicokinetic study is calculated to be between one-half and one-quarter of the total daily exposure at the MTD based on normal feed or water consumption and bodyweight while the other doses are fractions of the top dose. Using the model, plasma concentrations are predicted for the planned feed or drinking water study. A feed or drinking water toxicokinetic study is then conducted and blood samples are taken over the course of a 7- to 14-day exposure period. If the determined plasma concentrations fall in the range of those achieved after gavage dosing, which themselves resulted in dose proportional concentrations, then the dosed-feed/water concentrations are assumed to be in the dose- proportional range as well.

In support of inhalation studies, blood samples are taken after a 4- to 6-hour whole-body or nose-only exposure period. The steady-state plasma concentration and the elimination rate are determined. The steady-state concentrations are plotted versus exposure concentration to make the determination of dose proportionality. A kinetic model is developed and used to predict steady-state concentrations and the time to reach steady state.

Multiple exposure studies may include obtaining information on possible enzymatic inhibition or induction effects as well as the possibility of bioaccumulation. In such studies, animals are dosed by gavage (or other appropriate route) for 14 days with one dose, usually the highest anticipated dose; sufficient animals are used so that three data points are available at each blood sampling time. Blood samples are taken at multiple time points after dosing and analyzed for test chemical or metabolite. These results are compared to the results of the single- exposure definitive study to determine possible enzymatic inhibition or induction effects and bioaccumulation of the test chemical.

The third step is to conduct toxicokinetic studies in conjunction with the actual toxicology studies to estimate internal dose as well as possible changes in the kinetics after repeated dosing and aging. The doses used in the 2-year carcinogenicity study are chosen so that, if possible, the two lower doses are in the linear kinetic range to facilitate extrapolation to lower doses and interspecies scaling while the top dose is chosen to be the MTD.

Another challenge to the toxicokineticist involves relating any observed changes in kinetics during a long-term toxicology study to age effects. In an attempt to address this issue, the kinetics of the chemical in a control group of animals are determined at 18 months of a 2-year study. Extra animals are added to each dose group in the 2-year study and used at 2 weeks and at 3, 12, and 18 months of dosing so that it is possible to follow any changes in kinetic behavior as the dosing progresses.

An appendix is included that provides examples of four current toxicokinetic study protocols for chemicals scheduled for toxicological evaluation using oral (gavage and feed), topical, and inhalation routes.

ANALYTICAL METHOD VALIDATION

Validation of biological sample analysis methods is critical to the evaluation of the final data. A description of the validation procedure utilized by the NTP is provided here. The method needs to demonstrate the appropriate specificity, precision, absolute recovery, measurement limits, and relative error. An evaluation of the blank biological sample matrix contribution to responses seen in spiked samples also needs to be determined.

In conducting the validation, spiked biological sample standards are prepared at six different concentrations, using two independently prepared stock standards of different concentrations. Spiked biological sample standards at the lowest and highest concentrations are prepared in triplicate. Single spikes are prepared at the four intermediate concentrations. Biological sample blanks collected from five animals are prepared in triplicate. The spiked standards are prepared so that every other standard comes from one stock solution while the remaining standards are prepared from the second stock solution. The instrumental response from a single analysis of each biological standard and blank is then recorded. A series of solvent

standard solutions with the same final concentrations (i.e., after any extraction, dilution, or concentration step) are also prepared. The same standard stock solutions, prepared as described above, are used for making these solutions. A reagent blank in the same solvent as the standards is also prepared. Once all standards are prepared, the analytical response is determined and calculations made to evaluate the correlation coefficient for the solvent and biological sample standard curve data as well as the precision, percent relative standard deviation (percent RSD), the percent recovery (based on a comparison of responses of the biological and solvent standards), the relative error, and the measurement limits.

The limit of detection (LOD) is defined as three times the standard deviation (SD) of the blank response expressed as concentration. The limit of quantitation (LOQ) is defined as 10 times the SD of the blank response expressed as concentration. If there is no blank value, the SD of the lowest concentration should be used in the calculations.

Often another estimate of the LOQ is used, the experimental limit of quantitation (ELOQ). This value is defined as the lowest biological sample standard concentration that has been analyzed and that has a defined relative error and a defined RSD (e.g., 15 percent relative error and 15 percent RSD).

During the analysis of the actual study samples, a standard curve using a series of spiked biological sample standards is generated with each batch of samples. The standard curve is analyzed prior to any study samples. Once analysis of study samples has been initiated, quality control samples are run at fixed intervals throughout the analytical run. An acceptable frequency depends on the method being used, but a common approach is to run a quality control sample after every 10 study samples. Quality control samples are spiked biological sample standards that are prepared at the time the actual study samples are collected and stored under the same conditions as the study samples. Quality control samples are usually prepared at two or three concentrations that cover the expected study sample concentrations.

EXAMPLES

Over the last several years, the NTP has increasingly focused on the generation of toxicokinetic data and its application in toxicology study design and interpretation. Examples are presented to illustrate how NTP has utilized the toxicokinetic data.

3'-Azido-3'-deoxythymidine

3'-Azido-3'-deoxythymidine (AZT) is the first FDA-approved drug in the United States for the treatment of acquired immunodeficiency syndrome (AIDS). To aid in the design of a planned 2-year carcinogenicity study, the bioavailability and dose proportionality of AZT in B6C3F1 mice of both sexes were evaluated (Trang 1993). The chemical was administered IV or intragastrically at doses of 15, 30, and 60 milligrams per kilogram (mg/kg). There were no differences in the kinetics of AZT between the sexes. The elimination half-life was 18 minutes and the bioavailability was 85 percent. Dose proportionality was confirmed over the doses planned in the chronic study.

Benzyl Acetate

Benzyl acetate is widely used as a flavoring agent in the food industry and as a fragrant ingredient in a variety of consumer products such as soaps and lotions. The NTP conducted two 2-year carcinogenicity studies in F344 rats and B6C3F1 mice, one using the daily gavage administration and the other using dosed-feed administration. Although the daily doses of benzyl acetate were comparable between the two studies, hepatic tumorigenicity was only observed in the gavage studies of mice. In contrast, necrotic lesions were seen in the brain in both the feed and gavage studies. Comparative toxicokinetic studies were conducted in F344 rats and B6C3F1 mice to estimate the impact of gavage (500 mg/kg in rats and 1000 mg/kg in mice using corn oil as the vehicle) versus dosed-feed administration (dosed-feed pentachlorophenol concentrations provide a daily dose of 648 mg/kg for rats and 900 mg/kg for mice) on the toxicokinetics of benzyl acetate and to provide further information for interpreting the toxicity differences observed (Yuan et al. 1995). In an in vitro study, the half-life of benzyl acetate was found to be less than a minute; therefore, it was decided to monitor internal dose based on benzyl alcohol, hippuric acid, and benzoic acid plasma concentrations. In rats and mice, the benzoic acid plasma concentrations after gavage dosing were about a hundred times the concentrations found in a study in which benzyl acetate was administered in the feed. In both studies the benzyl alcohol plasma concentrations were less than 0.1 micrograms per milliliter (g/mL) while hippuric acid concentrations were about 20 percent of the benzoic acid concentrations. In the benzyl acetate study, results of the plasma analyses showed that the method of dosing had a major impact on the internal dose of the

metabolite, benzoic acid, which may explain the route-dependent differences in toxicity.

Cadmium Oxide

Cadmium oxide has many industrial uses, including anticorrosion coating for iron, copper, and steel. The lung burden and systemic exposure in male Fischer 344 rats were evaluated during a 13-week toxicology study in which the animals were exposed to aerosolized cadmium oxide at concen-trations of 0.1, 0.25, or 1.0 mg per cubic meter (mg/m^3) for 6 hours a day (Dill et al. 1994). Concentrations of cadmium were deter-mined in the blood, kidney, and lung during the course of the toxicology study. Accumulated cadmium in the lungs was not proportional to exposure concentration. Although the concentrations of cadmium in the blood were very low, the amount in the kidneys represented a significant fraction of the lung burden. Thus there was a significant systemic exposure after inhalation of the aerosolized cadmium oxide.

p-Chloro-,,-trifluorotoluene

p-Chloro-,,-trifluorotoluene (CTFT) is a widely used chemical intermediate in the manufacture of dinitroaniline herbicides. Various routes of administration were considered. The inhalation route was excluded because of the expense involved in the conduct of inhalation studies. The chemical's volatility excluded the conduct of a dermal or dosed-feed study, while its insolubility in water excluded the conduct of a drinking water study. Therefore a gavage study was selected. Corn oil was considered as the vehicle but administration of corn oil has been related to increases in the incidences of pancreatic lesions in male F344 rats. Therefore a new approach was considered, that is, molecular encapsulation of CTFT with a-cyclodextrin (CD), which might then be soluble in water. To evaluate the acceptability of this approach a toxicokinetic study was conducted in male Fischer 344N rats (Yuan et al. 1991*a*). Animals were dosed IV at 4.7 mg/kg using a 10 percent Tween 80 aqueous solution, and intragastrically at 10, 50, and 400 mg/kg doses in either corn oil or CD-complex in water. Bioavailability was shown to be complete for both vehicles and dose proportionality was established up to at least 400 mg/kg. It was concluded that CD could be used in the planned toxicology studies of CTFT.

Codeine

Codeine is an opioid that is an effective analgesic and antitussive therapeutic agent. NTP conducted a 2-year carcinogenicity study in Fischer 344 rats of both sexes with codeine administered in the feed (400, 800, and 1,600 parts per million (ppm)). Toxicokinetic information from rats in the 2-year study was developed for comparison to human data to serve as one basis for extrapolating the results from the rodent study to humans (Yuan et al. 1992, 1994*c*). Blood samples were collected from the rats on days 7, 21, and 90 at 7:00 pm, 11:00 pm, 3:00 am, and 7:00 am. Additional collections were made at 16 and 24 months between 6:00 and 8:00 am. Plasma concentrations of codeine and its metabolite, morphine, were determined. Bioavailability of codeine using the dosed feed route increased with dose (from 10 to 25 percent). The concentrations of codeine in rats receiving 800 ppm in the feed were comparable with the mean concentration reported in humans receiving a 60 mg oral dose, while the determined concentrations of morphine conjugates in the rat are much higher than reported in humans. The presence of morphine conjugates might have a significant impact on the interpretation of the rat codeine 2-year toxicology study.

2',3'-Dideoxycytosine

2',3'-Dideoxycytosine (DDC) is a drug approved for the treatment of AIDS. Plasma concentrations of DDC were determined at the conclusion of the 180-day short-term toxicology study, and the kinetic profile of DDC was determined for B6C3F1 and NIH Swiss mice. The incidence of thymic lymphoma found in this 6-month study correlated directly to internal dose as measured by area under the plasma versus time curve, but not the administered dose. This finding was applied to both strains of mice studied (B. Collins, personal communication, September 12, 1994).

2'3'-Dideoxyinosine

2'3'-Dideoxyinosine (DDI) is a therapeutic agent used in the treatment of AIDS. DDI is known to be easily hydrolyzed in the presence of acid. To aid in the selection of a vehicle for administration of DDI in planned toxicological evaluations, the bioavailability of DDI was determined in B6C3F1 mice of both sexes after intragastric administration in buffered and unbuffered formulations (R. Handy, personal communication, January 11, 1994). The significant effect of vehicle pH on the absorption of DDI was demonstrated by comparison of plasma DDI concentrations from buffered versus unbuffered oral dose formulations. The bioavailability

was 60 percent from buffered aqueous formulations and only 10 percent from unbuffered formulations.

o-Nitroanisole

o-Nitroanisole is widely used as a dye intermediate. A toxicology study was planned with the chemical administered in feed. In preliminary work to develop a method for confirmation of the accuracy of dose formulations, it was found that the complete recovery of the chemical from feed formulations required more severe extraction conditions as the feed formulations aged. There was concern that the increased strong binding of the chemical to feed constituents might affect the bioavailability in toxicology studies. To evaluate the bioavailability of o-nitroanisole from feed formulations, the urinary concentrations of o-nitroanisole's main metabolites, free and conjugated o-nitrophenol, were determined in 7-day dosed-feed studies conducted in male F344 rats using freshly prepared and aged dosed-feed formulations (Yuan et al. 1991*b*). No differences were found in the extent of bioavailability from the fresh and aged feed.

Oxazepam

Oxazepam is a widely prescribed benzodiazepine antianxiety agent and a common metabolite of many other benzodiazepines including diazepam and chlordiazepoxide. To aid in the assessment of risks associated with human use of this drug, the comparative toxicokinetic studies were conducted in F344 rats, B6C3F1 mice, and Swiss-Webster mice of both sexes after an IV dose of 20 mg/kg and oral gavage doses of 50, 200, and 400 mg/kg (Yuan et al. 1994*b*). In addition, since the NTP 2-year toxicology study used dosed feed, the toxicokinetics of oxazepam were also investigated in a 3-week dosed-feed study in male B6C3F1 mice at 125 and 2,500 ppm. Results indicated that the elimination of oxazepam from plasma after IV injection in both rats and mice were first-order and could best be described by a two-compartment model with a terminal elimination half-life of 4 to 5 hours for rats and 5 to 7 hours for mice. At all doses studied, the females had significantly higher plasma concentrations than males. At 50 mg/kg the bioavailability of oxazepam in rats (< 50 per-cent) was lower than in Swiss-Webster mice (> 80 percent). Based on the maximum plasma concentration achieved (Cmax), dose proportionality was not observed in rats or mice after gavage dosing. In the B6C3F1 mouse dosed-feed study, plasma concentrations increased proportionally with concentration of oxazepam in feed.

The estimated relative bioavailability from dosed feed (relative to the gavage study at 50 mg/kg) was about 43 percent.

Pentachloroanisole

Pentachloroanisole (PCA) has been found to be an environmental pollutant even though it has no major industrial uses. It is postulated that PCA is derived from methylation of pentachlorophenol, a widely known environmental pollutant. Since no internal dose determinations were conducted during the NTP-sponsored 2-year toxicology studies in B6C3F1 mice and F344 Fischer rats in which some evidence for carcino-genic potential was found, a retrospective toxicokinetic study was conducted to evaluate its basic kinetics as well as its potential for bioac-cumulation (Yuan et al. 1993). It this toxicokinetic study, B6C3F1 mice and F344 Fischer rats of both sexes were administered PCA intravenously (10 mg/kg) and intragastrically (10, 20, and 40 mg/kg). Both PCA and pentachlorophenol plasma concentrations were determined. PCA was shown to be rapidly demethylated to pentachlorophenol in both species, and the resulting pentachlorophenol plasma concentrations were in the range of one hundred times higher than those found for the parent PCA. Bioavailability of PCA was only about 36 percent in rats but was about 74 percent in mice. Plasma concentrations increased with dose, but were not directly proportional. No sex differences were observed. Significant potential for bioaccumulation of pentachlorophenol from exposure to PCA was predicted.

Pentachlorophenol

Pentachlorophenol is an effective broad-spectrum biocide widely used as a wood preservative. Two-year carcinogenicity studies had been conducted in B6C3F1 mice and similar studies were planned in Fischer 344 rats. To aid in future comparison of the results of the toxicology studies in both species and to provide information for dose-response relationships, toxicokinetic evaluations were conducted. In single- and multiple-exposure studies the toxicokinetics of pentachlorophenol were studied in the Fischer 344 rat using IV and oral (gavage and dosed-feed) routes of exposure (Yuan et al. 1994*a*). The elimination kinetics of pentachlorophenol after IV administration of 5 mg/kg were independent of sex. After gavage administration, dose proportionality was established up to at least 38 mg/kg. To aid in establishing dose-response relationships, the bioavailability of pentachlorophenol administered in dosed feed was calculated and compared to that after gavage dosing. The

bioavailability of pentachlorophenol administered in dosed feed at concentrations of 302 and 1,010 ppm ranged from 30 to 52 percent, which was significantly lower than bioavailability of pentachlorophenol administered by gavage (86 to 100 percent). The time course of pentachlorophenol plasma concentrations during the dosed-feed study was simulated using a computer model based on linear theory (Yuan 1993). The simulations were comparable with the experimentally determined concentrations.

SUMMARY

Toxic responses to test chemicals are known to be dependent on the exposure route, the kinetic behavior of the chemical, and the dose used in the toxicology study. Therefore, knowledge of internal dose is indispensable for the interpretation of toxicology study results, for the facilitation of interspecies scaling, and for risk assessment. By monitoring the blood and/or tissue concentrations of test chemical and/or metabolites versus time after administration of study chemicals by different routes, the bioavailability and kinetic characteristic of test chemicals can be readily obtained. This data can define the so-called linear dose range using area under the plasma concentration versus time curve, clearance, or other related toxicokinetic parameters, and can also be used to predict the possible bioaccumulation under multiple dose regimes. Changes in kinetic parameters after multiple exposures indicate alteration in how the animal handles the chemical (e.g., that there was enzyme induction or inhibition).

A recommended approach for conducting toxicokinetic studies generally involves three steps. Step 1 is a preliminary study, which uses a minimum number of animals to estimate the range of blood/tissue concentrations, the required quantitation limit for the analytical method, and the optimal sampling times for the definitive toxicokinetic studies. Step 2 is the definitive study and generates blood and/or tissue concentration data for calculating the toxicokinetic parameters. Step 3 is the toxicokinetic study conducted in conjunction with the toxicology study to determine the internal dose and the effects of age and continuous exposure on kinetic parameters.

Examples of the application of NTP toxicokinetic evaluations were also presented in this chapter, demonstrating their use in the design and interpretation of toxicology studies.

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Appendix:
Examples of NTP Toxicokinetic Protocols

TOXICOKINETICS STUDY PROTOCOL FOR METHYLENE BLUE (AN ORAL STUDY USING INTRAGASTRIC DOSING)

Rationale

The toxicokinetic studies are designed to: determine the basic kinetics including dose proportional range and bioavailability, provide an estimate of the internal dose, and provide information about long-term exposure on the kinetics.

Preliminary Study

Analytical Method. A preliminary analytical method shall be developed for quantitation of test chemical in plasma over the target concentration range of 0.1 to 50 g/mL.

Preliminary IV Study. Groups of 12 male Fisher 344N rats and female B6C3F1 mice (13Å2 weeks old) shall be dosed intravenously with test chemical (2.5 mg/kg for rats and 2.5 mg/kg for mice). The preferred vehicle for IV formulation is Emulphor/ethanol/water (1:1:8). The volumes used for dosing by the IV route will be 2 mL/kg for rats and 4 mL/kg for mice. As much heparinized blood as possible shall be collected from the vena cava from one dosed animal at each of 12 time points (3, 5, 10, 20, 30, and 40 min; 1, 1.5, 2, 3, 4, and 6 hours). Test chemical concentrations in plasma shall be determined using the unvalidated analytical method.

Preliminary Gavage Study. Groups of 12 Fisher 344N rats and B6C3F1 mice of both sexes (13Å2 weeks old) shall be gavaged at one of two doses of test chemical (2.5 and 50 mg/kg for rats and 2.5 and 25 mg/kg for mice). The vehicle for the gavage formulation will be 0.5 percent aqueous methylcellulose. The volumes used for dosing by the gavage route will be 5 mL/kg for rats and 10 mL/kg for mice. As much heparinized blood as possible shall be collected from the vena cava from one dosed animal at each of 12 time points (5, 10, 20, 30, and 45 min; 1, 1.5, 2, 3, 4, 6, and 8 hours). Test chemical concentrations in plasma shall be determined using the unvalidated analytical method.

General Information. Confirmatory dose formulation analyses shall *not be* conducted in the preliminary IV and gavage studies, but *shall be* conducted for the dosed-feed studies. Pooled blank plasma from rats shall be used to construct the standard curves for each preliminary study if no species differences are noted in the method using blank plasma.

Method Performance Evaluation. If the preliminary study results are satisfactory and quantitation of test chemical in plasma is in a range for which quantitation is technically feasible, then the analytical method shall be validated over the range dictated by the preliminary study results. Meanwhile, a stability study of the analyte in plasma shall be conducted over a period of time covering the expected storage time of the biological samples. The concentration used in the stability study shall be the midpoint concentration of the standard curve.

Single Administration Study

Rat IV Route. Groups of 12 Fisher 344N rats of both sexes (13Å2 weeks old) shall be dosed intravenously with a test chemical IV formulation at one dose (2.5 mg/kg). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after IV administration, with three animals bled at each time point. Two blood samples at different times (preferably >2 hr apart) shall be collected via alternating orbital sinuses from each dosed rat. Test chemical concentrations shall be determined in plasma using a validated analytical method.

Mouse IV Route. Groups of 24 B6C3F1 mice of both sexes (13Å2 weeks) shall be dosed intravenously with a test chemical IV formulation at one dose (2.5 mg/kg). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after IV administration, with three animals bled at each time point. Each mouse shall be bled once. Test chemical concentrations shall be determined in plasma using a validated method.

Rat Gavage Route. Groups of 12 Fisher 34N rats of both sexes (13Å2 weeks old) shall be gavaged at each of three doses of test chemical (2.5, 25, 50 mg/kg). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after gavage administration, with three animals bled at each time point. Two blood samples at different times (preferably >2 hr

apart) shall be collected via alternating orbital sinuses from each dosed rat. Test chemical concentrations shall be determined in plasma using a validated analytical method.

Mouse Gavage Route. Groups of 24 B6C3F1 mice of both sexes (13Å2 weeks old) shall be gavaged at each of three doses of test chemical (2.5, 12.5, 25 mg/kg). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after gavage administration, with three animals bled at each time point. Each mouse shall be bled once. Test chemical concentrations shall be determined in plasma using a validated analytical method.

General Information. Confirmatory dose formulation analyses shall be conducted for the single administration studies. Pooled control plasma from rats shall be used to construct the standard curves if no species differences are noted in the blank plasma.

Two-Year Studies

Special Study Animals. Special groups of 10 rats and 10 mice of both sexes shall be added to each dose group of the 2-year study. No control rats or control mice are needed for this study. For rats, blood samples shall be collected at 3, 6, 12, and 18 months using orbital sinus bleeding, with five rats being bled at each of two time points (to be specified by NTP). Rats shall be returned to their cages after the 3-, 6-, and 12-month bleeding intervals and sacrificed after the 18-month interval. Test chemical concentrations shall be determined in plasma using a validated analytical method.

For mice, blood samples shall be collected only at 12 months with five mice being bled at each of two time points (to be specified by NTP). After sampling, mice shall be sacrificed. Test chemical concentrations shall be determined in plasma using a validated analytical method.

Aged Rat Study. Using the sentinel rats in the 2-year study, 15 male and 15 female rats (18 months old) shall be gavaged at the middle dose. Blood samples shall be taken at five time points (to be specified by NTP), with three animals bled at each time point. Each rat shall be bled only once. Test chemical concentrations shall be determined in plasma using a validated analytical method.

Aged Mouse Study. Using the sentinel mice in the 2-year study, 15 male and female mice (18 months old) shall be gavaged at the middle dose. Blood samples shall be taken at five time points (to be specified by NTP), with three animals bled at each time point. Each mouse shall be bled only once. Test chemical concentrations shall be determined in plasma using a validated analytical method.

TOXICOKINETICS STUDY PROTOCOL FOR 2-HYDROXY-4-METHOXYBENZOPHENONE (AN ORAL STUDY USING DOSED FEED)

Rationale

The toxicokinetic studies are designed to: determine the basic kinetics including dose proportional range and bioavailability, provide an estimate of the internal dose, and provide information about long-term exposure on the kinetics.

Preliminary Study

Analytical Method. A preliminary analytical method shall be developed for quantitation of test chemical in plasma over the target concentration range of 0.1 to 50 g/mL.

Preliminary IV Study. Groups of 10 Fisher 344N rats and B6C3F1 mice of both sexes (13Å2 weeks old) shall be dosed intravenously with test chemical (8 mg/kg for rats and 50 mg/kg for mice). The preferred vehicle for IV formulation is Emulphor/ethanol/water (1:1:8). The volumes used for dosing by the IV route will be 2 mL/kg for rats and 4 mL/kg for mice. As much heparinized blood as possible shall be collected from the vena cava from one dosed animal at each of 10 time points (3, 5, 10, 20, and 40 min; 1, 2, 3, 4, and 6 hours). Test chemical concentrations in plasma shall be determined using the unvalidated analytical method.

Preliminary Gavage Study. Groups of eight Fisher 344N rats and B6C3F1 mice of both sexes (13Å2 weeks old) shall be gavaged at one of two doses of test chemical (8 and 80 mg/kg for rats and 50 and 500 mg/kg for mice). The vehicle for the gavage formulation will be 0.5 percent aqueous methylcellulose. The volumes used for dosing by the gavage route will be 5 mL/kg for rats and 10 mL/kg for mice. As much heparinized blood as possible shall be collected from the vena cava from one dosed animal at each of 8 time points (5, 15, 30, and

45 min; 1, 2, 4, and 8 hours). Test chemical concentrations in plasma shall be determined using the unvalidated analytical method.

Preliminary Feed Study. Groups of eight Fisher 344N rats and B6C3F1 mice of both sexes (13Å2 weeks old) shall be dosed with the test chemical in the feed for up to 7 days (time to be dosed will be based on the preliminary IV study results) at one of two dosed-feed concentrations (1,000 and 10,000 ppm). As much heparinized blood as possible shall be collected from the vena cava from one dosed animal at each of seven time points (4 am, 6 am, 8 am, 10 am, 12 pm, 4 pm, and 6 pm).

General Information. Confirmatory dose formulation analyses shall *not be* conducted in the preliminary IV and gavage studies, but *shall be* conducted for the dosed feed studies. Pooled blank plasma from rats shall be used to construct the standard curves for each preliminary study if no species differences are noted in the method using blank plasma.

Method Performance Evaluation. If the preliminary study results are satisfactory and quantitation of test chemical in plasma is in a range for which quantitation is technically feasible, then the analytical method shall be validated over the range dictated by the preliminary study results (determined by NTP) per the current NTP general specifications requirements. Meanwhile, a stability study of the analyte in plasma shall be conducted over a period of time covering the expected storage time of the biological samples. The concentration used in the stability study shall be the midpoint concentration of the standard curve.

Single Administration Study

Rat IV Route. Groups of 12 Fisher 344N rats of both sexes (13Å2 weeks old) shall be dosed intravenously with a test chemical IV formulation at one dose (to be chosen based on results of the preliminary study). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after IV administration, with three animals bled at each time point. Two blood samples at different times (preferably > 2 hr apart) shall be collected via alternating orbital sinuses from each dosed rat. Test chemical concentrations shall be determined in plasma using a validated analytical method.

Mouse IV Route. Groups of 24 B6C3F1 mice of both sexes (13Å2 weeks) shall be dosed intravenously with a test chemical IV formulation at one dose (to be chosen based on results of the preliminary study). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after IV administration, with three animals bled at each time point. Each mouse shall be bled once. Test chemical concentrations shall be determined in plasma using a validated method.

Rat Gavage Route. Groups of 12 Fisher 34N rats of both sexes (13Å2 weeks old) shall be gavaged at each of three doses of test chemical (to be chosen based on results of the preliminary study). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after gavage administration, with three animals bled at each time point. Two blood samples at different times (preferably >2 hr apart) shall be collected via alternating orbital sinuses from each dosed rat. Test chemical concentrations shall be determined in plasma using a validated analytical method.

Mouse Gavage Route. Groups of 24 B6C3F1 mice of both sexes (13Å2 weeks old) shall be gavaged at each of three doses of test chemical (to be chosen based on results of the preliminary study). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after gavage administration, with three animals bled at each time point. Each mouse shall be bled once. Test chemical concentrations shall be determined in plasma using a validated analytical method.

General Information. Confirmatory dose formulation analyses shall be conducted for the single administration studies. Pooled control plasma from rats shall be used to construct the standard curves if no species differences are noted in the blank plasma.

Two-Year Studies

Special Study Animals (GLP). Special groups of 10 rats and 10 mice of both sexes shall be added to each dose group of the 2-year study. No control rats or control mice are needed for this study. For rats, blood samples shall be collected at 3, 6, 12, and 18 months using orbital sinus bleeding, with five rats being bled at each of two time points (to be specified by NTP). Rats shall be returned to their cages after the 3-, 6-, and 12-month bleeding intervals and sacrificed after

the 18-month interval. Test chemical concentrations shall be determined in plasma using a validated analytical method.

For mice, blood samples shall be collected only at 12 months, with five mice being bled at each of two time points (to be specified by NTP). After sampling, mice shall be sacrificed. Test chemical concentrations shall be determined in plasma using a validated analytical method.

Aged Rat Study. Using the sentinel rats in the 2-year study, 15 male and 15 female rats (18 months old) shall be gavaged at the middle dose. Blood samples shall be taken at five time points (to be specified by NTP), with three animals bled at each time point. Each rat shall be bled only once. Test chemical concentrations shall be determined in plasma using a validated analytical method.

Aged Mouse Study. Using the sentinel mice in the 2-year study, 15 male and female mice (18 months old) shall be gavaged at the middle dose. Blood samples shall be taken at five time points (to be specified by NTP), with three animals bled at each time point. Each mouse shall be bled only once. Test chemical concentrations shall be determined in plasma using a validated analytical method.

TOXICOKINETICS STUDIES FOR CAMPHOR (A TOPICAL EXPOSURE)

Rationale

The camphor toxicokinetic studies using the topical exposure route are designed to determine: absolute bioavailability and basic kinetics, percent of internal dose from nontopical exposure, the extent of bioaccumulation, the internal dose, and the effects of age and long-term exposure on the kinetics.

Preliminary Study

Analytical Method Development. A preliminary analytical method shall be developed for quantitation of camphor in plasma over the target concentration range of 0.5 to 50 g/mL.

Preliminary IV Study. Groups of 10 rats and 10 mice of each sex (13Å2 weeks old purchased by the contractor) shall be dosed intravenously with camphor at 6 and 50 mg/kg for rats and mice,

respectively. Emulphor/ethanol/water (1:1:8) is to be used as the vehicle. The volumes used for dosing by the IV route will be 2 mL/kg for rats and 4 mL/kg for mice. As much heparinized blood as possible shall be collected from the vena cava from one dosed rat and mouse at each of 10 time points (5, 15, 30, and 45 min; 1, 2, 4, 6, 8, and 10 hrs). Camphor concentrations in plasma shall be determined using the unvalidated analytical method.

Preliminary Topical Exposure Study. Groups of eight rats and eight mice of each sex (13Å2 weeks old) shall be topically exposed to camphor in ethanol/water//1/1 at two dosages (25 and 400 mg/kg for rats and 200 and 1,000 mg/kg for mice). The volumes used for dosing by the topical route shall be the same as in chronic study (i.e., 0.6 mL/kg for rats and 2.0 mL/kg for mice). The concentrations of the dose formulations are to be kept constant. The site of application shall be protected to avoid grooming by the rodent but the site shall not be occluded. As much heparinized blood as possible shall be collected from the vena cava from one dosed rat and mouse at each of 10 time points (15, 30, and 45 min; 1, 2, 4, 8, 12, 16, and 24 hrs). Camphor concentrations in plasma shall be determined using the unvalidated analytical method.

General Information. Confirmatory dose formulation analyses shall *not be* conducted in the preliminary IV or topical exposure studies. Pooled control plasma from male and female rats shall be used to construct the standard curves for each preliminary study.

Method Performance Evaluation. If the preliminary study results are satisfactory and quantitation of camphor in plasma is in a range for which quantitation is technically feasible, then the analytical method shall be validated over the range dictated by the preliminary study results (determined by NTP). Meanwhile, a stability study of the analyte in plasma shall be conducted over a period of time covering the expected storage time of the biological samples. The concentration in plasma evaluated in the stability study shall be 75 percent of the highest concentration used for the standard curve.

Single Administration Study

Rat IV Route. Groups of 12 male and 12 female rats (13Å2 weeks old) shall be dosed intravenously with a camphor IV formulation at one dose (to be chosen based on results of preliminary study). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after IV administration, with

three animals bled at each time point. Two blood samples at different times (preferably >2 hr apart) shall be collected via alternating orbital sinuses from each dosed rat. Camphor concentrations in plasma shall be determined using the validated analytical method.

Mouse IV Route. Groups of 24 male and 24 female mice (13Å2 weeks old) shall be dosed intravenously with a camphor IV formulation at one dose (to be chosen based on results of the preliminary study). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after IV administration, with three animals bled at each time point. Camphor concentrations in plasma shall be determined using the validated analytical method.

Rat Topical Exposure Route. Two groups of 12 male and 12 female rats (13Å2 weeks old) shall be exposed to camphor at three doses (to be chosen based on results of the preliminary study). One group will have the site of application protected to prevent grooming by the rodents while the second group will not have the site protected. Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after topical exposure, with three animals bled at each time point. Two blood samples at different times (preferably >2 hr apart) shall be collected via alternating orbital sinuses from each dosed rat. Camphor concentrations in plasma shall be determined using the validated analytical method.

Mice Topical Exposure Route. Two groups of 24 male and 24 female mice (13Å2 weeks old) shall be exposed to camphor at three dosages (to be chosen based on results of the preliminary study). One group will have the site of application protected to prevent grooming by the rodents while the second group will not have the site protected. Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after topical exposure, with three animals bled at each time point. Camphor concentrations in plasma shall be determined using the validated analytical method.

General Information. Confirmatory dose formulation analyses shall be conducted for the single administration studies. Pooled control plasma from male and female rats shall be used to construct the standard curves.

Chronic Studies

Special Study Animals (GLP). Special groups of 10 rats and 10 mice of both sexes shall be added to each core dose group of the chronic study. No control rats or control mice are needed for this study. For rats, blood samples shall be collected at 3, 6, 12, and 18 months using orbital sinus bleeding, with two rats being bled at each of five time points (to be specified by NTP). Rats shall be returned to their cages after the 3-, 6-, and 12-month bleeding intervals and sacrificed after the 18-month interval. Plasma concentrations of camphor shall be determined.

For mice, blood samples shall be collected only at 12 months, with two mice being bled at each of five time points (to be specified by NTP). After sampling, mice shall be sacrificed. Plasma concentrations of camphor shall be determined.

Aged Rat Study. Using the sentinel rats in the chronic study, 15 male and 15 female rats (18 months old) shall be dosed at the middle dose. Blood samples shall be taken at five time points (to be specified by NTP) after topical exposure, with three animals bled at each time point. Each rat shall be bled only once. Plasma concentrations of camphor shall be determined.

Aged Mouse Study. Using the sentinel mice in the chronic study, 15 male or female mice (18 months old) shall be dosed at the middle dose. Blood samples shall be taken at five time points (to be specified by NTP) after topical exposure, with three animals bled at each time point. Each mouse shall be bled only once. Plasma concentrations of camphor shall be determined.

TOXICOKINETICS STUDIES PROTOCOL FOR DECALIN/TETRALIN (AN INHALATION EXPOSURE)

Rationale

The NTP toxicokinetic studies are designed to: determine the kinetics of decalin/tetralin in blood, determine the internal dose during inhalation studies as well as any possible changes in kinetics, establish dose proportional range, provide information on the effect of age on the kinetic parameters, and determine the concentrations of known metabolites in kidneys (only for the rat decalin studies).

Preliminary Studies

Analytical Method. A preliminary analytical method shall be developed for quantitation of decalin/tetralin in blood. In the decalin studies methods are also to be developed for the determination of the concentration of known metabolites (cis-2-decalone and trans-2-decalone) in rat kidneys. This range shall be estimated from literature and previous experience.

Preliminary IV Study. Groups of 10 rats and 10 mice of each sex (13Å2 weeks old purchased by the contractor) shall be dosed intravenously with decalin/tetralin at two doses proposed by the contractor and approved by NTP. Emulphor/ethanol/water (1:1:8) solutions shall be used for IV administration. The volumes used for dosing by the IV route will be 2 mL/kg for rats and 4 mL/kg for mice. As much heparinized blood as possible shall be collected from the vena cava from one dosed rat and mouse at each of 10 time points (5, 10, 20, and 40 min; 1, 2, 4, 6, 8, and 12 hours). Blood concentrations of decalin/tetralin shall be determined using the unvalidated analytical method.

Concentrations of the two decalin ketone metabolites are to be determined in the kidneys from the 1-, 4-, 8-, and 12-hour time point in rats of both sexes.

Preliminary Inhalation Study. Groups of five rats and five mice of each sex (13Å2 weeks old purchased by the contractor) shall be exposed for 6 hours to decalin/tetralin vapor via inhalation route at each of two exposure concentrations. The exposure concentrations shall be set after the completion of the 14-day toxicology study and shall be the highest and lowest concentrations proposed for the 90-day study. Blood samples shall be taken from five animals per sex per specie immediately after shutdown of the exposure (not to exceed 10 minutes). Samples shall be analyzed to estimate the possible steady-state concentration range.

Concentrations of the two decalin ketone metabolites are to be determined in the rat kidneys of both sexes.

General Requirement. Confirmatory analyses of the dose formulation shall *not be* conducted in the preliminary study. Pooled control blood from male and female rats shall be used to construct the standard curves for *each* preliminary study.

Validating the Method. If the preliminary study results are satisfactory and quantitations of decalin/tetralin in blood are in a technically feasible range, then the analytical methods shall be validated over the range dictated by the preliminary study results (determined by NTP). A stability study of the analytes in blood shall be conducted over a period of time covering the expected storage time of the biological samples. The samples are to be analyzed as soon as possible but not to exceed 3 weeks. The concentrations used in the stability study shall be the *midpoint* concentration of the standard curve.

Single Administration Study

Rat IV Route. Groups of three jugular-cannulated rats of each sex (13Å2 weeks old purchased by the contractor) shall be dosed intravenously with a decalin/tetralin IV formulation at each of two doses (to be chosen based on results of preliminary study and the results of the 90-day inhalation toxicology study). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after IV administration, with three animals bled at each time point. Blood concentrations of decalin/tetralin shall be determined using the validated analytical method.

Concentrations of the two ketone metabolites of decalin are to be determined in the rat kidneys of both sexes at four time points (to be determined by NTP).

Mouse IV Route. Groups of 12 mice (13Å2 weeks old purchased by the contractor) of each sex shall be dosed intravenously with a decalin/ tetralin IV formulation at each of two doses (to be chosen based on results of the preliminary study and the 90-day inhalation toxicology study). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after IV administration, with three animals bled at each time point. Each mouse shall be sampled twice via alternating orbital sinuses at different times (preferably > 2 hr apart). Blood concentrations of decalin/tetralin shall be determined using the validated analytical method.

Rat Inhalation Route (Single Exposure). Groups of 12 rats (13Å2 weeks old purchased by the contractor) of each sex shall be exposed to decalin/tetralin vapor for 6 hours at each of three exposure concentrations via inhalation route. The exposure concentrations

shall be based on the results of the 90-day toxicology study. Immediately after shutdown of the exposure, the decline of blood concentrations of decalin/tetralin in rats shall be followed at eight time points (to be determined based on the results of the preliminary study), with three animals bled at each time point. The first time point shall be as close as possible to time zero. Time zero is defined as the time of shutdown of the chamber exposure. Each rat shall be sampled twice via alternating orbital sinuses at different times (preferably > 2 hr apart). Blood concentrations of decalin/tetralin shall be determined using the validated analytical method.

Concentrations of the two ketone metabolites of decalin are to be determined in the rat kidneys of both sexes at four time points (to be determined by NTP).

Aged Rat Inhalation Route. Groups of 24 aged rats (18 months old purchased by the contractor) of each sex shall be exposed to decalin/ tetralin vapor for 6 hours using the proposed *middle* chronic exposure concentration via inhalation route. Immediately after shutdown of the exposure, the decline of blood concentrations of decalin/tetralin shall be followed at eight time points (to be determined based on the results of the preliminary study), with three animals bled at each time point. The first time point shall be as close as possible to time zero. Time zero is defined as the time of shutdown of the chamber exposure. Each rat shall be sampled only once. Blood concentrations of decalin/ tetralin shall be determined using the validated analytical method.

Mouse Inhalation Route (Single Exposure). Groups of 12 mice (13Å2 weeks old purchased by the contractor) of each sex shall be exposed to decalin/tetralin vapor for 6 hours at each of three exposure concentrations via inhalation route. The exposure concentrations shall be based on the results of the 90-day toxicology study. Immediately after shutdown of the exposure, the decline of blood concentrations of decalin/tetralin in mice shall be followed at eight time points (to be determined based on the results of the preliminary study), with three animals bled at each time point. The first time point shall be as close as possible to time zero. Time zero is defined as the time of shutdown of the chamber exposure. Each mouse shall be sampled twice via alternating orbital sinuses at different times (preferably > 2 hr apart). Blood concentrations of decalin/tetralin shall be determined using the validated analytical method.

Aged Mouse Inhalation Route. Groups of 24 aged mice (18 months old purchased by the contractor) of each sex shall be exposed to decalin/tetralin vapor for 6 hours using the proposed *middle* chronic exposure concentration via inhalation route. Immediately after shutdown of the exposure, the decline of blood concentrations of decalin/tetralin in mice shall be followed at eight time points (to be determined based on the results of the preliminary study), with three animals bled at each time point. The first time point shall be as close as possible to time zero. Time zero is defined as the time of shutdown of the chamber exposure. Each mouse shall be sampled only once. Blood concentrations of decalin/tetralin shall be determined using the validated analytical method.

General Requirement. Confirmatory dose formulation analyses for the IV studies shall be conducted for the single administration studies. Pooled control blood from male and female rats shall be used to construct the standard curves.

Chronic Studies

Special Study Animals. If the single administration study is successful, then groups of nine rats of each sex shall be added to each dose group of the chronic study. No control rats are needed for this study. Blood samples shall be collected at 2 weeks and 3, 6, 12, and 18 months using orbital sinus bleeding, with three rats being bled at each of six time points (to be determined based on the single administration study). Each rat shall be sampled twice via alternating orbital sinuses at different times (preferably >2 hr apart). Rats shall be returned to their cages after being sampled and sacrificed after the 18-month bleedings. Blood concentrations of decalin/tetralin shall be determined using the validated analytical method.

Groups of nine mice of each sex shall be added to each dose group of the chronic study. No control mice are needed for this study. Blood samples shall be collected only at 12 months with three mice being bled at each of six time points (to be determined based on the single-administration study). Each mouse shall be sampled twice via alternating orbital sinuses at different times (preferably >2 hr apart). After sampling, mice shall be sacrificed. Blood concentrations of decalin/tetralin shall be determined using the validated analytical method.

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