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Strategies to assess systemic exposure of chemicals in subchronic/chronic diet and drinking water studies ☆

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Abstract

Strategies were developed for the estimation of systemically available daily doses of chemicals, diurnal variations in blood levels, and rough elimination rates in subchronic feeding/drinking water studies, utilizing a minimal number of blood samples. Systemic bioavailability of chemicals was determined by calculating area under the plasma concentration curve over 24 h (AUC-24 h) using complete sets of data (≥5 data points) and also three, two, and one selected time points. The best predictions of AUC-24 h were made when three time points were used, corresponding to C_{max}, a mid-morning sample, and C_{min}. These values were found to be 103 ± 10% of the original AUC-24 h, with 13 out of 17 values ranging between 96 and 105% of the original. Calculation of AUC-24 h from two samples (C_{max} and C_{min}) or one mid-morning sample afforded slightly larger variations in the calculated AUC-24 h (69 – 136% of the actual). Following drinking water exposure, prediction of AUC-24 h using 3 time points (C_{max}, mid-morning, and C_{min}) was very close to actual values (80 – 100%) among mice, while values for rats were only 63% of the original due to less frequent drinking behavior of rats during the light cycle. Collection and analysis of 1 – 3 blood samples per dose may provide insight into dose-proportional or non-dose-proportional differences in systemic bioavailability, pointing towards saturation of absorption or elimination or some other phenomenon warranting further investigation. In addition, collection of the terminal blood samples from rats, which is usually conducted after 18 h of fasting, will be helpful in rough estimation of blood/plasma half-life of the compound. The amount of chemical(s) and/or metabolite(s) in excreta and their possible use as biomarkers in predicting the daily systemic exposure levels are also discussed. Determining these parameters in the early stages of testing will provide critical information to improve the appropriate design of other longer-term toxicity studies.

Keywords: Systemic exposure; Oral bioavailability; Diurnal variation; Steady-state blood level; Subchronic dietary studies; Subchronic drinking water studies; Dietary versus bolus gavage dose

Introduction

Biological effects of a chemical are best correlated with their concentration in the blood or plasma (systemically available dose) rather than the administered dose (e.g., gavaged, dermally applied, provided in food or water) (Chasseaud, 1992; Monro, 1992). Not all the administered dose is necessarily systemically bioavailable, and thus correlating an outcome to the total dose administered provides less meaningful information than correlating it to the dose present in blood/plasma or at the target organ(s). This is especially true for subchronic and chronic toxicity studies where a steady-state blood concentration is relatively quickly achieved (within ~5 half-lives of the chemical), with diurnal concentration variations in the central compartment (i.e., blood or plasma) correlating with the feeding/drinking pattern, distributional effects, and/or the elimination half-life. Therefore, determination of the area under the plasma or blood concentration–time curves over 24 h (AUC-24 h), after steady-state levels are achieved, is relevant for estimating the daily internal exposure of a chemical.

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Estimating the maximum and minimum blood concentrations (C_{max} and C_{min}) during dietary exposure and comparing them with the results of equivalent bolus oral dosing also will be valuable. The C_{max} values may play a critical role in expression of toxicity, and blood C_{max} concentrations after bolus dosing may be higher than those observed after similar mg/kg dietary exposure. The comparison of C_{max} to C_{min} is indicative not only of the daily concentration fluctuations in the central (blood) compartment but also potentially at the target organs, thus providing a better understanding of the toxicity profile of a chemical. In addition, such pharmacokinetic information may be useful in determining non-dose-proportional increases and/or decreases in blood/plasma concentration of a chemical resulting from saturation of absorption, first-pass metabolism, induction or inhibition of metabolism, or saturation of clearance. Knowledge of non-dose-proportional changes in AUC-24 h in the early stages of testing will be helpful in selecting study doses that will support the rational design of safety testing for the chemical, as has been suggested by an International Life Science Institute (ILSI) project panel (Timchalk, 2003).

Establishing a procedure for accurately estimating blood/plasma AUC-24 h and C_{max} and C_{min} values from minimum blood samples will enable the addition of these determinations to ongoing subchronic/chronic toxicity studies. To avoid compromising results of ongoing diet or drinking water study, addition of such assessments can only be practically achieved if daily systemic dose is calculated from a limited number of blood samples collected over 24 h (1–3 samples). Alternatively, a group of three to four satellite animals can be added to each dose group for specific collection of blood samples in order to avoid additional stress to the test animals. Adding satellite animals also will potentially allow achieving these goals in smaller rodent species (e.g., mice). The design of traditional subchronic rat studies also permits a comparison of blood concentrations at steady-state levels with that at the time of sacrifice (which is routinely conducted after 16–18 h of fasting). Such comparisons will provide rough estimates of elimination kinetics of the parent compound and/or the known metabolite(s) that can be useful in designing pharmacokinetic and/or absorption, distribution, metabolism, and elimination studies.

Few studies have described methods to estimate plasma concentration–time profiles over 24 h at steady state, ranging from computer modeling of the data obtained from single dose studies to serial and sparse sampling (Burtin et al., 1996; Jochemsen et al., 1993; Mahmood, 1997; Nedelman et al., 1993; Tse and Nedelman, 1996; Yuan, 1993). Burtin et al. (1996) have described the use of a single blood sample from each animal to calculate AUC and C_{max}. While this approach represents a minimally invasive approach, a more accurate measurement of AUC may be obtained with additional sampling. Using a limited data set of 3 drugs with half-lives ranging from 1 to 5 h, Jochemsen et al. (1993), after sampling animals every 3 h over the course of a 24 h period, determined that only three blood samples taken at selected times from each animal during a 24 h period would accurately estimate AUC and C_{max}.

Determination of excretion, especially in urine, of parent and/or major metabolite(s) over 24 h during the course of the exposure will allow potential relationships between the excreted amounts and the circulating levels of analyte(s) in blood to be established. Such data will be useful in refining pharmacokinetic models for estimating systemic exposure of a chemical from the amount of analyte(s) found in urine. Improved methods for estimating systemic exposure levels gathered from human non-invasive urinary biomonitoring studies will facilitate improved comparisons to internal dose determinations of animals undergoing hazard-based chemical testing, thus providing valuable data-based refinements to the risk assessment process. In addition, the amount of compound recovered in urine over 24 h will provide an estimate of the daily systemic exposure showing actual daily systemic exposure either from occupation, environment, or mobilization from the depot within the body (e.g., fat, bones). Once data have been generated for a number of diverse molecules and trends established, this non-invasive procedure can be more broadly conducted both in animals and humans.

The objective of this study was to develop a practical method to determine the steady-state daily systemic exposure of a chemical to animals in subchronic dietary/drinking water toxicity studies by determining the area under the blood/plasma concentration time curve (AUC-24 h) for compounds of different blood/plasma half-life. The approach involved collection of a comprehensive number of blood samples within a 24 h period and then examining various combinations of 1–3 time points predictive of the actual AUC values. This information provides a scientific basis (e.g., sampling times in reference to light/dark cycle) for choosing the minimum number and best combination of time points in order to predict the daily systemic bioavailability of a chemical (AUC-24 h) as well as daily highest (C_{max}) and lowest (C_{min}) body burden and the time of their occurrence. This study also investigated the use of terminal blood concentrations from a rat subchronic study collected after ~18 h of fasting/removal of fortified diet to roughly estimate elimination half-life of chemicals, as well as the use of time-course urinary elimination as an indicator of the circulating blood levels/AUC-24 h at steady-state exposure levels.

Materials and methods

In order to better characterize the blood/plasma and urine AUC of chemicals with different pharmacokinetic profiles, dietary administration studies were conducted at our facility with 2,4-dichlorophenoxyacetic acid (2,4-D) (short plasma half-life; Sauerhoff et al., 1977; Pelletier et al., 1989; van

Ravenzwaay et al., 2003) and chlorpyrifos (relatively long plasma half-life of its metabolite trichloropyridinol; Timchalk et al., 2002). These prototype chemicals were selected due to limited metabolism, availability of extensive toxicity and metabolism data, and established methods of analysis for parent compound and major metabolite(s).

Data of diurnal blood variations for a number of additional chemicals (dichloroacetic acid, benzyl acetate, doxazosin, acetaminophen, antipyrine, phenylbutazone, pentachlorophenol, cyclohexylamine–HCl, and saccharin) were collected from the literature (Sweatman and Renwick, 1980; Roberts and Renwick, 1989; Charuel et al., 1992; Jochemsen et al., 1993;Yuan et al., 1994, 1995; Saghir and Schultz, 2002; Schultz et al., 2002) and analyzed for AUC using the same approach described in this report.

In a separate proof-of-concept study conducted at our facility, three blood samples (5 am, 10 am, and 5 pm) were collected from the tail vein of male rats (n = 5/dose) 24 days after the initiation of dietary exposure to a chemical (pesticide) under development (X574175) and analyzed. An additional blood sample was collected from the orbital sinus of each rat at the time of sacrifice (~18 h after the withdrawal of exposure) to roughly estimate elimination half-life. Terminal serum samples were also collected and analyzed from a mouse (n = 3/dose/gender) subchronic dietary study involving the same chemical. Results of these studies are discussed with reference to the practical implications of the proposed scheme of blood collection and interpretations/usefulness.

Chemicals and dose preparation. Chlorpyrifos (2,3,5-trichloro-6-diethoxyphosphinothioyloxy-pyridine), 2,4-D (2,4-dichlorophenoxyacetic acid), and X574175 were obtained from Dow AgroSciences, LLC (Indianapolis, Indiana). Chemical purity of chlorpyrifos, 2,4-D, and X574175 was 97.6, 97.3, and 95%, respectively. The test diets were prepared by serially diluting concentrated chemical-feed mixtures (premix) with ground feed. Premixes were prepared once and used for the duration of the experiments. The chemicals were stable for the experimental period. Diets were prepared weekly based upon the most recent body weight and feed consumption data. Initial concentrations of chemicals in the diet were calculated using historical body weights and feed consumption data. Aliquots of fortified diet were analyzed for the chemicals by extracting in suitable solvent and analyzing by LC/MS/MS. Homogeneity of chemicals in feed mixtures was determined once, prior to the start of each study.

Animals. Young adult animals were purchased from Charles River Laboratories Inc. (Raleigh, NC) and allowed to acclimatize to the animal facility for 1 week prior to the dietary exposure. They were housed in rooms designed to maintain adequate conditions (22 ± 3 °C, 40–70% relative humidity, 12 h light/dark photocycle, air exchange 12–15 times/h) and provided with LabDiet Certified Rodent Diet #5002 (PMI Nutrition International, St. Louis, Missouri). Feed and municipal water were provided ad libitum. After acclimatization, animals were randomly assigned to dosing groups. The Institutional Animal Care and Use Committee of The Dow Chemical Company approved the experimental protocols.

Dietary dosing of 2,4-D and chlorpyrifos. Groups of 48 male Fischer 344 rats (9 weeks old, ~200 g) per chemical were fed chemical-fortified diets for 4 weeks. The target doses for 2,4-D were 5 and 100 mg/kg/day. These dose levels were chosen based on the results of 2,4-D feeding studies (Charles et al., 1996). The low dose is 3-fold lower than the subchronic No-Observed-Effect-Level (NOEL) and equals the overall rat chronic dietary NOEL. The high dose produces some toxicological effects after 13-week dietary exposure, e.g., decreased feed consumption and reduction in body weights (Charles et al., 1996), and represents the subchronic Low-Observed-Effect-Level (LOEL) dose in rats. The target doses for chlorpyrifos were 0.5 and 5.0 mg/kg/day. The low dose of 0.5 mg/kg/day was chosen as a dose where minimal cholinesterase inhibition is anticipated and detectable levels of chlorpyrifos were expected following 4 weeks of dietary exposure. The high dose of 5 mg/kg/day was chosen as a dose where extensive plasma cholinesterase inhibition would be anticipated with minimal signs of cholinergic toxicity (Mattsson et al., 2000).

Rats were housed individually in stainless steel cages during the study, and food consumption was recorded. The percentage of chemical in diet was converted to mg/g diet and then to actual dose of mg/kg body weight/day using the feed consumption and body weight data of each animal. At the end of 4th week (26–27th day), four rats from each dose group were moved to identical cages with urine separation and collection equipment. Animals were fed treated diet during the urine collection period; special precautions were taken to avoid contamination of urine with treated diet. Urine from these rats was collected over dry ice at 6 h intervals starting approximately 6 am and continued for 24 h. After completion of the urine collection, the rats were moved to their original cages. On day 29, groups of three rats were sacrificed every 3 h starting at 6 am (Fig. 1). Rats sacrificed during the dark period (between 6 pm to 6 am) were kept in separate rooms with dark cycle in order to maintain the photocycle. Rats were sacrificed at 6 am, 9 am, noon, 3 pm, 6 pm, 9 pm, midnight, and 3 am. Food consumption for every time interval was recorded to correlate consumption of chemicals to blood levels of parent chemical or metabolite. Rats were sacrificed by CO_2, and blood was collected by cardiac puncture. Concentration of chlorpyrifos and/or its metabolites (chlorpyrifos-oxon and 3,5,6-trichloro-2-pyidinol [TCP]) was determined in the weighed aliquots of whole blood and urine using the method described by Mattsson et al. (2000). Concentration of 2,4-D was determined in plasma and urine using LC/MS as described by van Ravenzwaay et al. (2003).

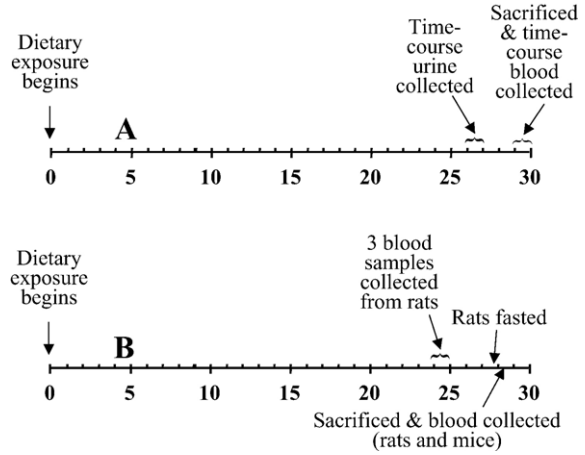

Fig. 1. Study design showing the start of the dietary exposure and time-course urine and blood collection for 2,4-D and chlorpyrifos (A) and X574175 (B).

Data from proof-of-concept rat and mouse subchronic studies. From a rat subchronic dietary study of a pesticide (X574175) under development, blood samples from male rats (n = 5/dose) were collected at 5 am, 10 am, and 5 pm, 24 days after the initiation of the study. Blood samples ($\leq$100 μl) were collected from the tail vein into heparinized mini-vials, and plasma obtained by centrifugation. In addition, blood was also collected from anesthetized rats via orbital sinus of each dose group (n = 5/dose) at the time of terminal sacrifice following 18 h of fasting/removal of fortified diet (on 29th day after the initiation of the study) and serum obtained. X574175 was composed of two isomers (X517131 and X513999), and plasma and serum samples were analyzed for both of the components.

Serum samples were also collected from a mouse subchronic X574175 dietary study at the time of their sacrifice (n = 3 for each dose and gender), 28 days after the start of feeding fortified diet. In contrast to rats, mice are not fasted prior to their sacrifice, and therefore terminal serum concentrations among doses were used to determine proportionality and dose-dependent systemic bioavailability at steady-state exposure levels.

The actual dose (mg/kg/day) was calculated from the feed consumption and body weight data as described above for 2,4-D and chlorpyrifos. The doses of X574175 to male mice and rats were 8, 25, 69, and 188 mg/kg/day and 9, 39, and 154 mg/kg/day and to female mice were 10, 33, 94, and 246 mg/kg/day, respectively. The doses of X517131 and X513999 were calculated using their relative amounts (64% X517131 and 31% X513999) in the formulation and diet. Plasma and serum levels of both components were measured by LC/MS/MS to estimate systemic bioavailability of each component of X574175 at the given dietary doses. The time-course plasma concentrations in rats were used to determine diurnal variation at each dose level after reaching steady state and also to calculate AUC-24 h during the course of steady-state exposure. Dietary exposure of X574175 for 24 days was long enough to establish steady-state levels in the animals based on the reported elimination half-life ($t_{½,\beta}$) of 25–44 h for a related chemical in rats (unpublished data). Blood was purposefully sampled on day-24 instead of day-27 or day-28 to minimize the impact of sampling, if any, on pathological evaluations conducted on day-29.

For rats, concentration in the serum collected at the time of sacrifice (18 h after the removal of fortified diet on day-28) was used, in conjunction with the 5 pm day-24 plasma data (Fig. 1), for rough estimation of the rate of plasma elimination and half-life ($t_{½}$). This was done with the assumption that the concentrations of the chemicals (X517131 and X513999) in blood were similar at the time when fortified feeds were removed to that measured at 5 pm, 4 days prior to fasting due to steady-state levels with natural diurnal variations representing feeding pattern of rats. Although the fortified diets were removed at 3 pm on day-28, the blood concentrations were not expected to fluctuate substantially from steady-state blood levels measured at 5 pm due to long elimination half-life. Recovery of the isomers in plasma and sera prepared from fortified blood samples (n = 3) was found to be almost identical, and therefore the term plasma elimination half-life is used. For rats and mice, serum concentrations at the time of sacrifice were used to determine dose-proportionality as an indication of the systemic exposure.

Literature data. The time-course blood/plasma concentration data for a number of chemicals, both in rats and mice (n = 3–5), were obtained from published literature. In all cases, animals were exposed for one to several weeks prior to taking the blood/plasma samples except for pentachlorophenol, where the plasma samples were collected 5 days after the initiation of dietary administration. In all cases, a steady-state plasma concentration was established in the animals, and the data showed expected normal diurnal variations. Data from the published articles were obtained from the figures. The actual time-course plasma/blood concentration data were estimated from scanned data using the DigitizeIt computer application, v1.55 (BormiSoft2, Cologne, Germany). Theoretical log K_{ow} values were calculated with the computer application ACDLogD Suite (Advanced Chemistry Development, Inc. Toronto, Canada).

Data analysis. Descriptive statistical analysis (i.e., mean ± standard deviation) was performed using Microsoft Excel (Microsoft Corporation, Redmond, Washington). Mean values of these and literature obtained data were analyzed for AUC using all the time points as well as combination of 3, 2, and 1 time point values by trapezoidal method ($AUC_{0\rightarrow t}$) using Microsoft Excel. Whenever statistical analysis was needed to compare trends between doses and/or genders, data were analyzed using SAS statistical analysis computer software (SAS Institute Inc., Cary, North Carolina) through a two-way analysis of variance. Linearity

was determined graphically via regression analysis (as per Sweatman and Renwick, 1980; Roberts and Renwick, 1989) as well as by comparing ratios of X574175 components. For the rough estimation of plasma elimination half-lives of the components of X574175, plasma concentrations at 5 pm and the serum concentrations (which were identical to plasma) at the time of sacrifice were used. Both of the parameters were calculated using additional Microsoft Excel functions.

Results

The time-course plasma/blood concentrations of 2,4-D and chlorpyrifos are presented in Table 1. Similar data for 9 other chemicals obtained from the literature (17 sets of time-course plasma/blood concentration data from various concentrations in rats and mice) are presented in Table 2. These data show the diurnal variation in the plasma/blood concentrations of chemicals after reaching steady-state levels during subchronic/chronic exposure through diet or drinking water. A summary of the species/strain and gender of rodents exposed, duration of exposure before collecting 24-h blood samples, and matrix (plasma or blood) analyzed are shown in Table 3.

After dietary exposure to chlorpyrifos, no parent compound could be detected in blood above the limit of detection of 0.2 ppb, even at the highest dose of 5 mg/kg/day. Therefore, its terminal metabolite (TCP) was monitored. This was in contrast to the finding of parent compound (chlorpyrifos) in the blood of rats for up to 6 h after bolus oral gavage dose at the same dose level of 5 mg/kg (Mattsson et al., 2000; Timchalk et al., 2002). The difference in the systemic exposure to parent compound after bolus oral dosing versus dietary exposure indicates saturation of first-pass metabolism of chlorpyrifos upon gavage administration. Despite the indication of significant first-pass metabolism of chlorpyrifos, the plasma concentrations of TCP increased linearly with dose, suggesting no saturation of TCP formation or clearance over the range of test doses. In contrast to TCP, 2,4-D concentrations in plasma (Tables 1 and 3) and AUC values (Table 4) increased in a distinctly nonlinear fashion between the 5 and 100 mg/kg/day dietary doses. The nonlinear increase was apparent from an 89-fold increase in C_{max} and a 69-fold higher plasma AUC-24 h, far exceeding the expected 20-fold difference between doses. Higher than expected plasma AUC-24 h was also supported by the saturation of urinary excretion at the high dose; only 28-fold higher 2,4-D was recovered during 18 h of collection instead of 69-fold as expected from the difference in circulating levels (Table 5).

The light cycle that animals were exposed to during the course of various studies is also presented in Table 3. In all studies, animals were exposed to a light cycle of 12 h (either from 6 am to 6 pm or from 7 am to 7 pm) with the exception of two studies (cyclohexylamine–HCl and saccharin) where animals were exposed to a light cycle of 14 h (from 7 am to 9 pm). In almost all cases, the C_{max} of the chemicals was observed during the dark cycle, especially within 2–3 h prior to the scheduled time for the lights to be turned on. Similarly, in almost all cases, the C_{min} was during the end of light cycle, within 1–2 h before the lights were turned off (Table 3). In all of the feeding studies, the variation between C_{max} and C_{min} was generally 2- to 3-fold. However, the C_{max}/C_{min} variation in the drinking water exposure studies was higher (Table 3), with variations much higher (126-fold) in rats than mice (1- to 59-fold).

The chemicals described in this report varied significantly in lipophilicity. The octanol water partition coefficient (log K_{ow}) values ranged from 0.54 to 4.77 (Table 3). The variations between C_{max} and C_{min} were found to have a slight inverse correlation with the log K_{ow} of the chemical ($r^2 = -0.35$) (data not shown).

In a similar fashion, the relationship between elimination $t_{½}$ of the chemicals and C_{max}/C_{min} variability was explored. The elimination $t_{½}$ values for the studied compounds (obtained from the literature) varied from less than 10 min to more than 40 h (Table 3). Diurnal variations in plasma concentrations correlated poorly with the plasma elimination $t_{½}$ values ($r^2 = 0.15$) (data not shown). This lack of correlation between the plasma half-life and C_{max} and C_{min} variability is probably due to continuous low levels of food ingestion during the light cycle in addition to pronounced feeding activity during the start and end of dark cycle (Fig. 2) (Charuel et al., 1992).

The AUC values calculated by using all of the blood/plasma concentration values as well as selected 3, 2, and 1 time point data are shown in Table 4. The criteria to choose three time points from the whole set of data were based on obtaining the first sample close to the time when the lights were turned on, the second in mid-morning ~3 h after the lights are turned on (around 9 am), and the third late in the afternoon (2–3 h before the lights were turned off). The purpose of choosing these time points was to obtain data at

Table 1
Diurnal blood/plasma levels of chemicals and/or metabolite(s) (μg/ml) in male Fischer 344 rats following exposure via fortified diet after achieving steady-state levels (n = 3/time point)

Time	2,4-D				CPF[a]			
	5 mg/kg/day		100 mg/kg/day		0.5 mg/kg/day		5 mg/kg/day	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
12 am	0.59	0.17	29.54	4.90	0.10	0.01	0.82	0.16
3 am	0.62	0.06	36.08	4.76	0.10	0.01	1.06	0.06
6 am	0.72	0.15	63.95	6.02	0.08	0.01	0.92	0.05
9 am	0.48	0.03	50.27	9.76	0.08	0.02	0.56	0.01
12 pm	0.41	0.08	35.65	8.18	0.06	0.03	0.54	0.09
3 pm	0.42	0.34	38.64	14.12	0.05	0.00	0.77	0.19
6 pm	0.44	0.08	19.28	8.51	0.05	0.01	0.40	0.15
9 pm	0.64	0.04	23.54	8.86	0.09	0.03	0.99	0.12

2,4-D = dichlorophenoxyacetic acid; CPF = chlorpyrifos; SD = standard deviation (n = 3).
[a] Trichloropyridinol in the plasma of rats.

Table 2
Mean diurnal plasma levels of chemicals and/or metabolite(s) (μg/ml) following exposure via fortified diet or drinking water after achieving steady-state levels (data obtained from the published articles)

Time	Pentachlorophenol[a]		Benzyl acetate[b]		Doxazosin[c]			ACP[d]	AP[d]	PB[d]	CHH[e]		Saccharin[f]	DCA in drinking water[g,h]			
	ppm in diet		mg/kg/day		mg/kg/day			mg/kg/day	mg/kg/day	mg/kg/day	mg/kg/day		% in diet	g/L in drinking water			
	302	1010	648	900[i]	10	20	40	100	100	50	197	281[i]	5	0.2	0.05[i]	0.5[i]	2.0[i]
(*N*)	(3)	(3)	(2)	(2)	(4)	(4)	(4)	(5)	(5)	(5)	(7)	(7)	(3)	(4)	(3)	(3)	(3)
12 am					0.08	0.20	0.50				4.33	2.69	104.00	8.30	0.05	0.93	87.20
1 am								0.76	4.30	16.40							
2 am																0.23	37.60
3 am											5.58	3.56	92.70			0.35	42.60
4 am					0.11	0.24	0.56	0.98	4.30	17.50							51.10
5 am	30.60	79.26	94.60	34.90										11.37		0.44	36.30
6 am											6.29	2.94	118.00			0.28	
7 am	31.30	69.22	96.40	20.90				1.08	4.80	20.00				7.98	0.05		17.80
8 am					0.06	0.15	0.42										
9 am	37.10	77.04	92.80	30.10							5.27	2.84	98.20				4.24
10 am								0.54	3.00	10.10				2.50			1.49
11 am																	
12 pm	31.30	55.00			0.05	0.12	0.41				4.95	4.53	80.20	0.50			
1 pm								0.48	2.00	6.90							
2 pm	30.50	55.24												0.34			
3 pm											3.48	3.05	67.30				
4 pm	29.00	50.33			0.03	0.05	0.30	0.68	1.70	8.10				0.09			
5 pm														0.44			
6 pm			89.60	17.90							2.19	1.35	60.80				5.83
7 pm								0.88	1.90	6.90						0.15	14.10
8 pm	24.70	56.29			0.03	0.09	0.21							7.84	0.03		5.57
9 pm											1.39	3.12	78.50			0.37	17.80
10 pm	30.80	65.58						0.84	4.70	13.40					0.04	0.40	21.10
11 pm			97.60	32.20													

Data are mean of 2–7 animals. (*N*) = samples collected from specified number of animals at each time point; ACP = acetaminophen; AP = antipyrine; PB = phenylbutazone; CHH = cyclohexylamine hydrochloride; DCA = dichloroacetic acid

[a] Yuan et al., 1994.
[b] Hippuric acid in plasma (Yuan et al., 1995).
[c] Charuel et al., 1992.
[d] Jochemsen et al., 1993.
[e] Roberts and Renwick, 1989.
[f] Sweatman and Renwick, 1980.
[g] Dichloroacetic acid in rats (Saghir and Schultz, 2002).
[h] Dichloroacetic acid in mice (Schultz et al., 2002).
[i] Levels in blood or plasma of mice.

the C_{max} which usually occurred right before the lights were turned on, one at the midpoint between C_{max} and C_{min}, and the last to obtain data close to the C_{min} (Tables 1–3). For the calculation of AUC-24 h values using two data points, data were used from samples at the times of C_{max} and C_{min}. For calculation using a single sample, the mid-morning sample was chosen, which corresponds to a usual sacrifice time for animals on a subchronic/chronic studies. Table 4 shows some variation in the time chosen for these calculations due to unavailability of the data at the desired time points. In these cases, the closest sample was chosen.

Overall, the predicted AUC-24 h using only three selected time points was almost identical (103 ± 10 percent) to the values calculated using all time points (5–8) in animals (both rats and mice) exposed to the chemicals via diet. The three-point AUC values were between 88 and 136% of the actual AUC-24 h, with the predicted values for 13 out of 17 sets of data between 96 and 105% of the actual value (Table 4). The correlation between all and selected three-point AUC-24 h values in animals exposed through drinking water was slightly lower (82 ± 15%) with values of 63% and 80–100% for the rat and mouse, respectively.

The predicted AUC-24 h using two selected time point samples was 114 ± 43% of the AUC-24 h calculated using all time points in animals exposed to the chemicals via diet. Calculations using only 2 samples provided higher variability; values ranged from 71 to 261% of the actual (all time points) values; most (18 out of 21 sets of data) of the values were between 88 and 126%. Similarly, higher variation was observed among groups of animals exposed via drinking water; AUC-24 h predicted using two time points was between 76 and 146% of values calculated using all time points (Table 4).

Among the animals exposed to chemicals through diet, the predicted AUC-24 h using one selected time point

Table 3
Other relevant information regarding animal, dosing, matrix, analyte, light cycle, diurnal high and low blood/plasma concentration, and time of their occurrence

Compound	Species	Gender	Age at sampling	Dose	Length of dosing	Compound analyzed	Matrix	Light cycle	T_{max}	T_{min}	C_{max} (μg/ml)	C_{min} (μg/ml)	Variability (C_{max}/C_{min})	Plasma $t_{½}$ (h)[a]	Log K_{ow}[b]
Drinking water															
Dichloroacetic acid	F344	Male	~13 weeks	0.2 g/L in water	1 week	DCA	Plasma	6 am–6 pm	5 am	4 pm	11.37	0.09	126.33	1.6	0.54
Dichloroacetic acid	B6C3F1	Male	10 weeks	0.05 g/L in water	2 weeks	DCA	Blood	6 am–6 pm	7 am	8 pm	0.05	0.03	1.44	0.3	0.54
	B6C3F1	Male	11 weeks	0.5 g/L in water	2 weeks	DCA	Blood	6 am–6 pm	12 am	7 pm	0.93	0.15	6.19	0.3	0.54
	B6C3F1	Male	12 weeks	2.0 g/L in water	2 weeks	DCA	Blood	6 am–6 pm	12 am	10 am	87.20	1.49	58.52	0.3	0.54
Diet															
2,4-D	F344	Male	~13 weeks	5 mg/kg/day	4 weeks	2,4-D	Plasma	6 am–6 pm	6 am	12 pm	0.72	0.41	1.77	1.6	2.58
	F344	Male	~13 weeks	100 mg/kg/day	4 weeks	2,4-D	Plasma	6 am–6 pm	6 am	6 pm	63.95	19.28	3.32	1.6	2.58
Chlorpyrifos	F344	Male	~13 weeks	0.5 mg/kg/day	4 weeks	TCP	Blood	6 am–6 pm	3 am	3 pm	0.10	0.05	2.00	40.7	4.77
	F344	Male	~13 weeks	5 mg/kg/day	4 weeks	TCP	Blood	6 am–6 pm	3 am	6 pm	1.06	0.40	2.63	40.7	4.77
Pentachlorophenol	F344	Male	15 weeks	302 ppm	2.5 days	PCP	Plasma	7 am–7 pm	9 am	8 pm	37.10	24.70	1.50	5.6	4.78
	F344	Male	15 weeks	1010 ppm	2.5 days	PCP	Plasma	7 am–7 pm	5 am	4 pm	79.26	50.33	1.57	5.6	4.78
Benzyl acetate	F344	Male	11 weeks	648 mg/kg/day	1 weeks	Hippurate	Plasma	7 am–7 pm	11 pm	6 pm	97.60	89.60	1.09	0.2	1.93
	B6C3F1	Male	11 weeks	900 mg/kg/day	1 weeks	Hippurate	Plasma	7 am–7 pm	5 am	6 pm	34.90	17.90	1.95	0.1	1.93
Doxazosin	SD	Male	~12 weeks	10 mg/kg/day	8 weeks	Doxazosin	Plasma	7 am–7 pm	4 am	8 pm	0.11	0.03	3.67	1–2	0.65
	SD	Male	~12 weeks	20 mg/kg/day	8 weeks	Doxazosin	Plasma	7 am–7 pm	4 am	4 pm	0.24	0.05	4.80	1–2	0.65
	SD	Male	~12 weeks	40 mg/kg/day	8 weeks	Doxazosin	Plasma	7 am–7 pm	4 am	8 pm	0.56	0.21	2.67	1–2	0.65
Acetaminophen	F344	Male	29–45 weeks	100 mg/kg/day	3 weeks	Acetaminophen	Plasma	7 am–7 pm	7 am	1 pm	1.08	0.48	2.25	1.3	0.34
Antipyrine	F344	Male	29–45 weeks	100 mg/kg/day	3 weeks	Antipyrine	Plasma	7 am–7 pm	7 am	4 pm	4.80	1.70	2.82	3.1	0.27
Phenylbutazone	F344	Male	29–45 weeks	50 mg/kg/day	3 weeks	Phenylbutazone	Plasma	7 am–7 pm	7 am	7 pm	20.00	6.90	2.90	4.8	3.47
Cyclohexylamine–HCl	Wistar	Male	6 weeks	197 mg/kg/day	4 weeks	Cyclohexylamine	Plasma	7 am–9 pm	6 am	9 pm	6.29	1.39	4.53	2.0	1.4
	MF1 mice	Male	6 weeks	281 mg/kg/day	4 weeks	Cyclohexylamine	Plasma	7 am–9 pm	12 pm	6 pm	4.53	1.35	3.36	1.4	1.4
Saccharin	SD	Male	~19 weeks	5% in diet	9 weeks	Saccharin	Plasma	7 am–9 pm	6 am	6 pm	118.00	60.80	1.94	0.5	0.91

DCA = dichloroacetic acid; 2,4-D = dichlorophenoxyacetic acid; TCP = 3,5,6-trichloro-2-pyridinol; PCP = pentachlorophenol.

[a] Plasma/blood half-lives were obtained from the literature, mostly from the references cited in Table 2.

[b] Theoretical log K_{ow} values calculated for parent compounds using ACDLogD Suite (Advance Chemistry Development, Inc., Toronto, Canada).

Table 4
Area under the plasma/blood concentration curves calculated using complete data set presented in Tables 1 and 2 or using three, two, and one selected time points and their percent variations

Compound	Species	Gender	Dose	Analyte	AUC (μg h ml^{-1}) calculated using different set of data points							AUC (% of complete dataset)		
					All	3	Time	2	Time	1	Time	(All vs. 3)	(All vs. 2)	(All vs. 1)
Drinking water														
Dichloroacetic acid	F344	Male	0.2 g/L in water	DCA	133.48	84.00	7 and 10 am, 4 pm	100.97	7 am, 5 pm	60.07	10 am	62.93	75.64	45.00
Dichloroacetic acid	B6C3F1	Male	0.05 g/L in water	DCA	1.01	1.01	12 and 7 am, 8 pm	0.97	7 am, 8 pm	1.14	7 am	99.91	95.49	112.69
	B6C3F1	Male	0.5 g/L in water	DCA	7.61	6.07	5 and 6 am, 7 pm	7.74	6 am, 9 pm	6.65	6 am	79.85	101.76	87.40
	B6C3F1	Male	2.0 g/L in water	DCA	414.67	358.11	5 and 9 am, 6 pm	604.80	5 am, 7 pm	871.20	5 am	86.36	145.85	210.09
Diet														
2,4-D	F344	Male	5 mg/kg/day	2,4-D	12.95	13.08	6 and 9 am, 3 pm	13.92	6 am, 6 pm	11.54	9 am	101.02	107.54	89.18
	F344	Male	100 mg/kg/day	2,4-D	890.88	1207.51	6 and 9 am, 3 pm	998.79	6 am, 6 pm	1206.49	9 am	135.54	112.11	135.43
Chlorpyrifos	F344	Male	0.5 mg/kg/day	TCP	1.86	1.63	6 and 9 am, 3 pm	1.63	6 am, 6 pm	1.86	9 am	87.83	87.67	99.73
	F344	Male	5 mg/kg/day	TCP	18.14	18.84	6 and 9 am, 3 pm	15.89	6 am, 6 pm	13.34	9 am	103.86	87.62	73.53
Pentachlorophenol	F344	Male	302 ppm	PCP	732.00	752.00	7 and 9 am, 4 pm	672.00	7 am, 8 pm	890.40	9 am	102.73	91.80	121.64
	F344	Male	1010 ppm	PCP	1550.67	1488.69	7 and 9 am, 4 pm	1506.07	7 am, 8 pm	1848.97	9 am	96.00	97.12	119.24
Benzyl acetate	F344	Male	648 mg/kg/day	Hippurate	2245.60	2208.70	5 and 9 am, 6 pm	2210.40	5 am, 6 pm	2227.20	9 am	98.36	98.43	99.18
	B6C3F1	Male	900 mg/kg/day	Hippurate	649.35	636.40	5 and 9 am, 6 pm	633.60	5 am, 6 pm	722.40	9 am	98.01	97.57	111.25
Doxazosin	SD	Male	10 mg/kg/day	Doxazosin	1.44	1.51	4 and 8 am, 4 pm	1.60	4 am, 4 pm	1.54	8 am	104.89	111.33	106.90
	SD	Male	20 mg/kg/day	Doxazosin	3.40	3.31	4 and 8 am, 4 pm	3.49	4 am, 4 pm	3.53	8 am	97.38	102.66	103.79
	SD	Male	40 mg/kg/day	Doxazosin	9.61	10.05	4 and 8 am, 4 pm	10.40	4 am, 4 pm	10.03	8 am	104.62	108.28	104.41
Acetaminophen	F344	Male	100 mg/kg/day	Acetaminophen	18.72	19.29	7 and 10 am, 4 pm	23.52	7 am, 7 pm	12.96	10 am	103.04	125.64	69.23
Antipyrine	F344	Male	100 mg/kg/day	Antipyrine	80.10	74.55	7 and 10 am, 4 pm	80.40	7 am, 7 pm	72.00	10 am	93.07	100.37	89.89
Phenyl butazone	F344	Male	50 mg/kg/day	Phenylbutazone	297.90	310.50	7 and 10 am, 4 pm	322.80	7 am, 7 pm	242.40	10 am	104.23	108.36	81.37
Cyclohexylamine–HCl	Wistar	Male	197 mg/kg/day	Cyclohexylamine	100.44	116.87	6 and 9 am, 3 pm	101.76	6 am, 6 pm	126.48	9 am	116.35	101.31	125.93
	MF1 mice	Male	281 mg/kg/day	Cyclohexylamine	72.24	71.27	6 and 9 am, 3 pm	51.48	6 am, 6 pm	68.16	9 am	98.65	71.26	94.35
Saccharin	SD	Male	5% in diet	Saccharin	2099.10	2210.55	6 and 9 am, 3 pm	2145.60	6 am, 6 pm	2356.80	9 am	105.31	102.22	112.28

Data are mean of 2–7 animals. DCA = dichloroacetic acid; 2,4-D = dichlorophenoxyacetic acid; TCP = 3,5,6-trichloro-2-pyridinol; PCP = pentachlorophenol.

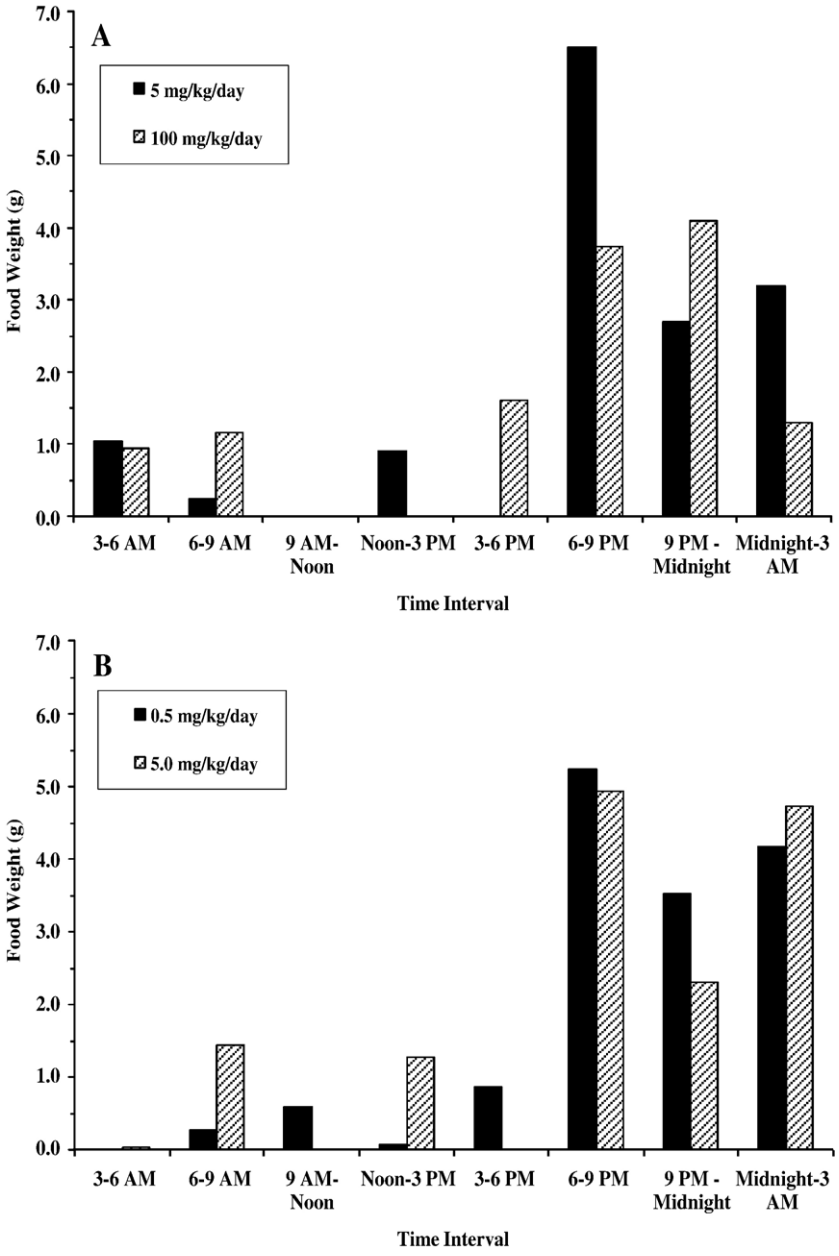

Fig. 2. Diurnal variations in the consumption of food by rats fed on 2,4-D (A) and chlorpyrifos (B) fortified diet. Each bar represents mean of 3 rats.

sample was 121 ± 56% of the AUC-24 h calculated using complete sets of time-course data (Table 4). The predicted AUC-24 h using a single time point was very close to the actual values when the sample used to predict the actual AUC-24 h was within 1–3 h after the lights were turned on. In the case of drinking water studies, samples in only one group of animals were taken 1 h after the lights were turned on, in all other groups, samples were taken either 1 h before, at the time, or 4 h after the lights were turned on. When the sample collected before the lights were turned on was used, the predicted AUC-24 h was 2-fold higher. When the sample collected 1 h after the lights were turned on was used, the predicted value was 113% of the actual value. Use of sample collected 4 h after the lights were turned on afforded predicted AUC-24 h values only 45% of actual (Table 4).

The AUC-24 h calculated from the data obtained by digitizing figures for doxazosin (Charuel et al., 1992), acetaminophen, antipyrine, phenylbutazone (Jochemsen et al., 1993), and cyclohexylamine–HCl (Roberts and Renwick, 1989) was compared with the reported AUC-24 h. No AUC-24 h values were available for other chemicals. The calculated AUC-24 h using digitized data was essentially identical to the reported values, with calculated values 100.8 ± 1.5% of the actual reported values. Jochemsen et al. (1993) have also reported AUC-24 h calculated by using 3 time points (7 am, 10 am, and 4 pm), similar to what we have used in Table 4, their values

matched with our calculations (100.2 ± 0.3%, ranging from 99.9 to 100.5%). Both of the parameters validated the data obtained by digitizing figures obtained from the literature.

Pilot use of the three time point data collection approach and terminal plasma level after 18 h of fasting in a conventional pesticide subchronic dietary study in rats

Diurnal plasma concentration variations of both of the isomers of X574175, after reaching steady-state level (24 days after the start of the rat feeding study), are shown in Fig. 3. At the two lower doses, the highest blood levels were found at 5 am and declined during the day, which is consistent with the feeding pattern of rats and observations from the dietary studies in Fig. 2 and Tables 1 and 2. The diurnal variation between C_{max} and C_{min} was only 1.1- and 1.8-fold for both of the doses, suggesting that steady state had been reached. For the high dose, the highest blood levels were found at the mid-morning sample (10 am). The mean AUC-24 h for X513131 was 182, 2385, and 15,441 ng/ml/h and for X513999 was 650, 3933, and 17,674 ng/ml/h at the low, middle, and high doses, respectively (Fig. 3). Even though animals were exposed to more than 2-fold higher levels of X517131 than X513999 based on their ratio in the formulation and diet, X513999 plasma concentrations were consistently higher than X517131. For X517131, the AUC-24 h increased nonlinearly with increasing dose; for X513999, AUC increased closer to linear over the range of tested doses (Fig. 3). The elimination of both of the components from plasma appeared saturated, particularly at the high dose which was apparent from the increasing plasma elimination half-lives with increasing dose.

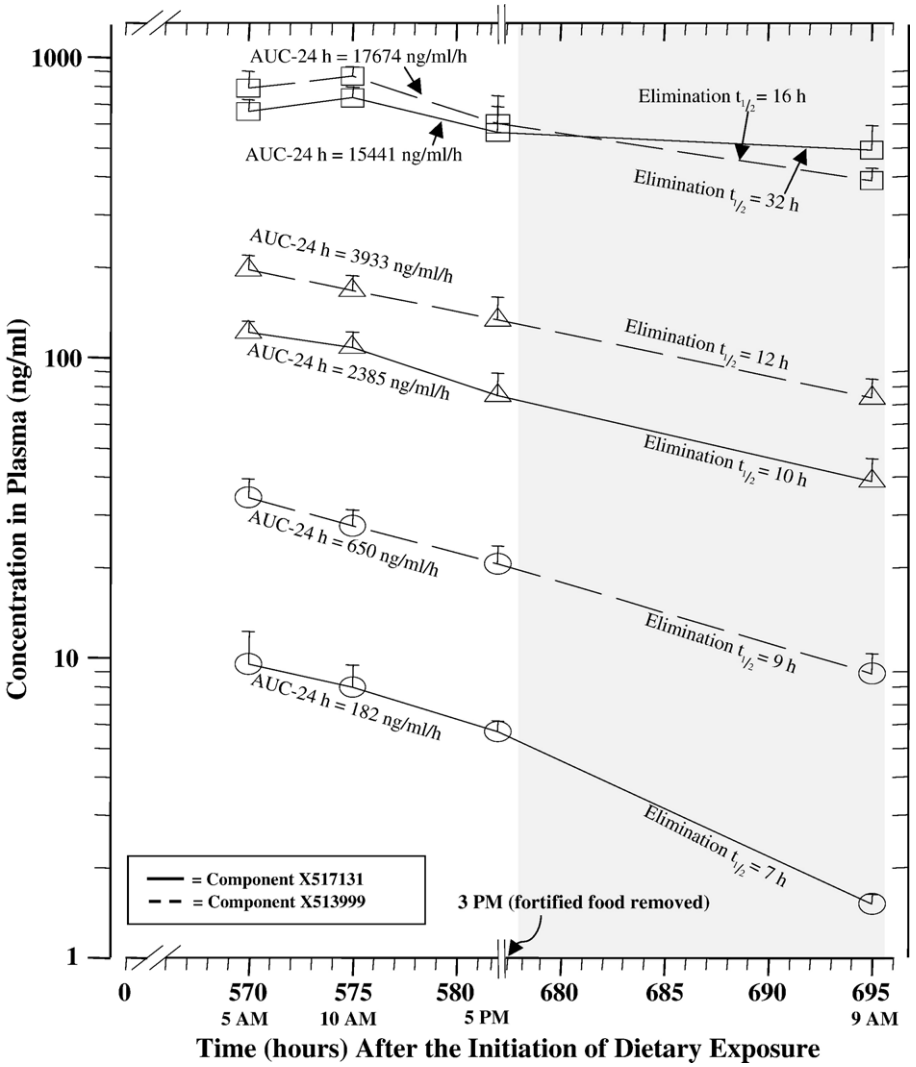

Fig. 3. Concentrations (ng/ml) of X513131 (solid lines) and X513999 (broken lines) in male rat plasma (mean ± SD, n = 5) after 24 days of administration of 0.12 (○), 0.50 (△), and 2.00 (□) mg/g X574175 in the diet showing diurnal variations (5 am, 10 am, and 5 pm at 570–682 h after initiation of dosing) at steady-state levels along with their respective AUC-24 h. Concentrations were also determined at sacrifice on test day 29 (695 h after the initiation of the study) in serum collected 18 h after the removal of the fortified diet (fasting) and used to calculate elimination half-lives (food withdrawn at 3:00 pm).

Pilot use of single time point data from mouse subchronic dietary study

As assessed by the serum concentrations of the components of X574175 at each dose level, systemic bioavailability as measured by AUC of X517131 was not statistically significantly different between male and female mice (Fig. 4; $P > 0.2$). However, systemic bioavailability of X513999 was statistically different ($P < 0.02$) between male and female mice, although this difference was likely mediated by the significantly reduced plasma concentrations of X513999 in female mice at the high dose. The decreased bioavailability of X513999 occurred even though females were exposed to ~24% higher amounts of test article due to higher feed consumption (Fig. 4). The increase in systemic bioavailability of X574175 in serum with increasing concentration of chemicals in the diet was linear for both of the compounds in male and female mice at the three lower doses ($r^2 = 0.99$ Fig. 4). Systemic bioavailability of both of the test components apparently became nonlinear at the highest dose (Fig. 4). The lower than dose-proportional increase in serum level at the highest dose was pronounced for X513999 in female mice, apparent from >40% lower than expected serum levels from the linear extrapolation of data from three lower doses and by the lower correlation coefficient ($r^2 = 0.91$) when data from all four doses were used (Fig. 4).

Use of urinary excretion data to determine internal exposure

The average amount of 2,4-D and TCP recovered in urine during 6 h collection intervals at the end of the 3-week

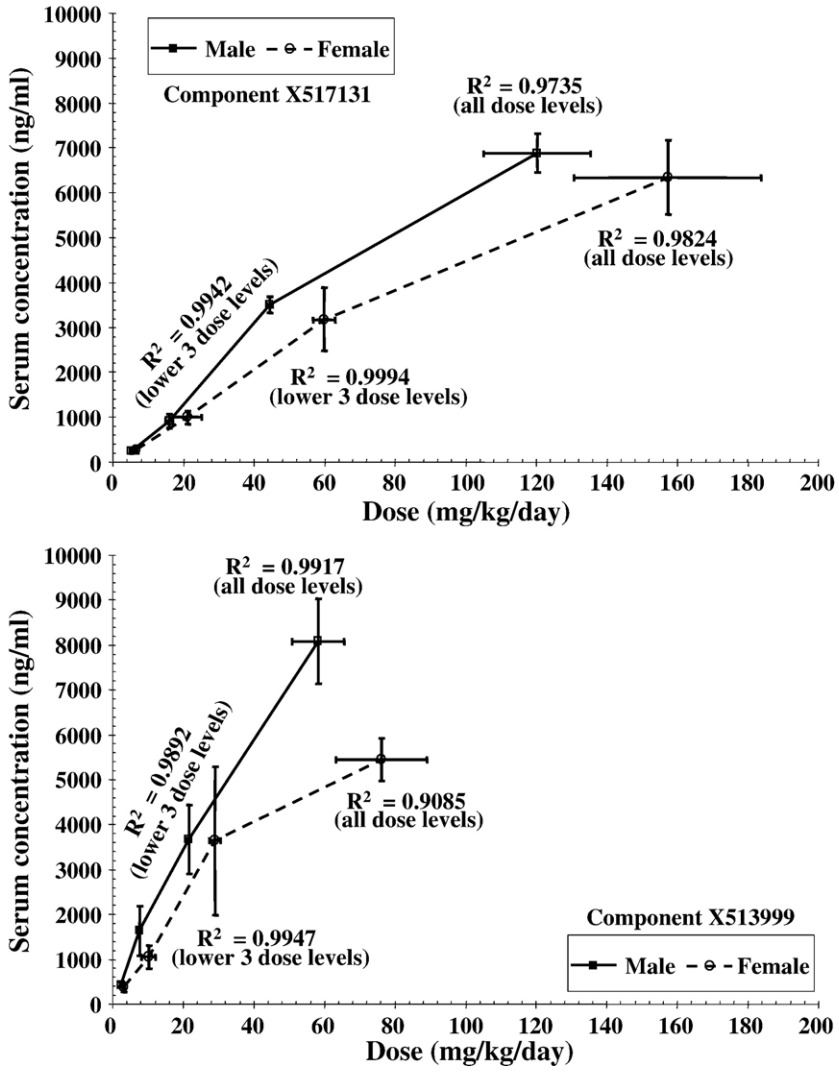

Fig. 4. Concentration (ng/ml) of X517131 and X513999 in mouse serum (mean ± SD, $n = 3$) at sacrifice following 28 days of administration of different levels of X574175 in the diet. Horizontal and vertical lines across the mean values represent standard deviations of the daily exposure of the chemical through diet and serum concentration among animals, respectively.

dietary studies are shown in Table 5. The pattern of elimination of the low dose of 2,4-D correlated with the levels found in blood. Slightly higher amounts of 2,4-D were excreted from 6 am to noon than noon to 6 pm and ~2-fold higher during the dark cycle, corresponding to lower and higher blood levels seen at steady state (Tables 1 and 5). Similar patterns of urinary excretion of the high dose of 2,4-D were observed (Table 5). The last urine samples (12 am–6 am) from the rats exposed to 2,4-D were not collected at the designated time and therefore not analyzed.

The time-dependent differences in urinary TCP levels of animals dosed with chlorpyrifos showed similar pattern at both doses; the least during the period from 6 am and noon when rats usually do not eat much, then levels started to increase later in the day corresponding to the feeding activities of rats (Fig. 2). The highest levels were found during the dark cycle (Table 5). Total amounts of TCP recovered in urine during 24 h at the low and high doses were 52 and 622 μg. Recovery of TCP at the high dose was 12-fold higher than the low dose (Table 5), close to the 10-fold difference in administered dose and the 9.8-fold difference observed between the plasma AUC-24 h values (Table 4). These data indicate that urinary elimination of TCP was not saturated at these two doses.

Table 5
Excretion of 2,4-D and TCP in rat (n = 4) urine after reaching steady-state blood levels by dietary exposure for 25 days[a]

Time	μg analyte/g urine		μg total analyte	
	Mean	SD	Mean	SD
2,4-D (5 mg/kg/day dietary exposure for 25 days)				
6 am–12 pm	71.86	19.07	144.77	22.25
12 pm–6 pm	75.13	18.67	125.91	21.70
6 pm–12 am	98.83	21.06	226.69	61.83
12 am–6 am	ND	ND	ND	ND
Mean	81.94	14.72	–	–
Total	–	–	497.37	53.58
2,4-D (100 mg/kg/day dietary exposure for 25 days)				
6 am–12 pm	1135.67	273.03	3313.50	539.42
12 pm–6 pm	1239.45	180.82	3138.34	739.38
6 pm–12 am	1829.49	253.55	7411.69	1197.82
12 am–6 am	ND	ND	ND	ND
Mean	1401.54	374.23	–	–
Total	–	–	13,863.53	2418.24
TCP (0.5 mg/kg/day dietary exposure of CPF for 25 days)[b]				
6 am–12 pm	2.81	0.31	11.65	3.56
12 pm–6 pm	3.41	0.14	11.77	1.40
6 pm–12 am	4.20	0.65	14.56	4.40
12 am–6 am	5.07	1.22	13.62	4.10
Mean	3.87	0.98	–	–
Total	–	–	51.59	1.43
TCP (5 mg/kg/day dietary exposure of CPF for 25 days)[b]				
6 am–12 pm	28.87	5.68	114.06	60.30
12 pm–6 pm	49.90	3.60	198.90	16.13
6 pm–12 am	52.73	5.32	170.22	44.79
12 am–6 am	51.38	13.16	138.58	30.15
Mean	45.72	11.29	–	–
Total	–	–	621.76	36.99

2,4-D = dichlorophenoxyacetic acid; TCP = trichloropyridinol (metabolite of chlorpyrifos [CPF]); SD = standard deviation (n = 4); ND = not determined (samples were not collected on time, therefore discarded).

[a] Rats were continued on dietary treatment during the 24 h urine collection period.

[b] Concentration of TCP in urine was corrected for the amount found in controls (1.40 ± 0.2 μg/g urine).

Discussion

It has been recognized for some time that correlating biological effects of a chemical to the systemically available dose (blood and/or plasma levels) is more meaningful than correlation to the nominal dose, especially in subchronic and chronic toxicity studies (Chasseaud, 1992; Jochemsen et al., 1993; Monro, 1992; Nedelman et al., 1993). Monro (1992) argued that, since the systemically available dose may be important for the outcome of an effect, it is better to use the absorbed dose, as estimated by AUC, C_{max}, and C_{min} concentrations, instead of administered dose for toxicity interpretations. Although the potential value of these data for effective interpretation of standard dietary or drinking water toxicity studies is well appreciated, the collection of such data has been hampered by a lack of understanding of appropriate sampling strategies that would reliably characterize AUC, C_{max}, and C_{min} values under standard toxicity test dosing conditions (usually diet or drinking water).

It is possible to collect multiple blood samples (6–8 samples/day) from larger animals (e.g., dogs, rabbits) on a study without compromising the toxicity results. However, it is not possible to do the same in rodent studies. Therefore, it is useful to evaluate different sampling strategies to achieve the goal of determining 24 h systemic bioavailability (AUC-24 h; C_{max}; C_{min}) of a chemical in subchronic and chronic studies after steady-state levels have been reached. A realistic design for rodent studies requires blood samples taken from animals be kept to a minimum number ($\leq$3 samples over 24 h) in order to avoid any negative impact on the outcome of the subchronic or chronic studies, while at the same time providing reasonable estimates of critical pharmacokinetic values.

This report demonstrates that the AUC-24 h, C_{max}, and C_{min} values for the plasma/blood curve in dietary and drinking water studies in rats and mice can be reasonably estimated using only a limited (preferably $\leq$3) number of samples when treated animals are at steady-state blood/plasma levels. The findings of this report, using environmental chemicals, also validate the approach of Jochemsen et al. (1993) in which use of three measurements of plasma concentration effectively estimated systemic exposures resulting from dietary treatment with short, intermediate, and long half-life pharmaceuticals.

In order to accurately predict AUC-24 h values using only three samples during a 24 h period, the timing of sampling was crucial (Jochemsen et al., 1993). Both the

study of Jochemsen et al. (1993) and the data in this report indicate that, for optimal kinetic analysis, the three samples should be taken 1 h before or right at the time when lights are turned on (C_{max}), in the mid-morning ~3 h after lights on (mid-range concentration), and lastly 1–2 h before the lights are turned off (C_{min}). For the test chemicals directly examined here and in other published studies, this sampling scheme predicted AUC-24 h values close (103 ± 10%) to the actual AUC-24 h calculated using 8–9 samples taken at a constant interval over a 24 h period. The data indicate that sample timing is critical in predicting AUC-24 h from a few time points, and variation by a few hours can afford significant changes in this kinetic parameter. Our findings regarding the impact of other time point combinations (data not reported) were similar to that of Jochemsen et al. (1993) who noted greater variation when time selective sampling was not coupled to the light cycle.

In almost all cases examined here (Table 3), the highest concentrations (C_{max}) of the chemicals in rats' blood/plasma were found a few hours before the lights are turned on. Similarly, the lowest concentrations of the chemicals (C_{min}) in rats were found a few hours before the lights were turned off. This was likely due to the nocturnal feeding habit of young rats used in these studies (Charuel et al., 1992; Roberts and Renwick, 1989, Sweatman and Renwick, 1980), an observation confirmed by food consumption monitoring in the current study (Fig. 2). In contrast to rats, timing of C_{max} and C_{min} in mice varied and did not follow any pattern with reference to the light and dark cycle (Table 3) as feeding/drinking patterns of mice are not as nocturnal compared to rats. The variability in mice suggests that a fixed sampling regimen may not be useful for estimation of C_{max} and C_{min} values, and effective sample times may require evaluation of diurnal feeding patterns, especially for exposure through drinking water. In the case of exposure through diet, the described schedule for collecting 3 blood samples for AUC-24 h in mice afforded 98–99% of the actual values (Table 4) even with the observed deviation from nocturnal feeding behavior when compared to rats.

When the chemicals were administered via diet, variation in the blood concentration (C_{max}/C_{min}) after reaching steady-state levels was between 1.1- and 4.8-fold in both species. The limited data available from drinking water studies indicate greater variation in C_{max} and C_{min} estimates (1.4- to 58.5-fold for mice and 126-fold for rats), possibly due to greater diurnal variation in drinking water consumption (Roberts and Renwick, 1989). Strategy for the estimation of AUC-24 h for drinking water exposure studies needs further evaluation using larger database. No correlation was observed between diurnal blood concentrations and plasma half-lives of the chemicals. However, the variation was much higher after exposure through drinking water.

Calculated AUC-24 h values using only two and one time point samples provided values reasonably close to the actual value, albeit with more variation. The decision to predict AUC-24 h using 2 or 1 sample(s) has to be taken very carefully as the time of sampling becomes more critical to overall variability. Thus, the data using a three time point sampling strategy as described will accurately predict AUC-24 h at steady state. This strategy can be implemented by either sampling core study animals, providing the sampling does not cause undue stress, or obtaining blood from additional satellite animals added to an ongoing subchronic or chronic study. Adding satellite animals will also allow collecting 3 samples from mice studies at designated times after the initiation of the studies.

Terminal blood samples in rat diet bioassays are routinely gathered after 16–18 h of fasting/removal of treated diet (mice are not fasted prior to terminal sacrifice). Sampling of blood while animals are exposed to treated diets coupled with terminal blood samples affords the opportunity to obtain rough estimates of elimination pharmacokinetics. Despite the approximate estimate of $t_{½}$ provided by only 2-time-point evaluation, these values nonetheless signaled potential nonlinear pharmacokinetic behavior in the data collected for the components of X574175 (Fig. 3). Thus, in addition to the plasma steady-state concentration data, the estimated decrease in plasma clearance with increasing dose provided confirmatory evidence of potential high-dose-dependent compromise of overall clearance rates.

It is important to recognize that an accurate estimate of plasma $t_{½}$ is essential to predict the time required to reach steady state in diet/drinking water studies. Thus, if plasma pharmacokinetic values are to be accurately estimated using the sampling strategy proposed in this report for 28-day (or greater) studies, preliminary data should be obtained indicating that test animals are indeed at steady state at the selected sampling times. It is expected that compounds having $t_{½}$ of $\leq$5 days would be at steady state after 28 days of dosing, and scenarios of not attaining steady state after 28 days of dosing would be very unlikely for vast majority of compounds.

Compared to rats, blood volumes required for chemical analyses in mice can limit serial sampling in dietary or drinking water toxicity studies. In those cases, however, non-fasted sampling of terminal blood/plasma/serum for chemicals of concern across the doses can still provide valuable information. Analysis of the single time point samples from the mouse study presented in this report (Fig. 4) suggests that the systemic bioavailability of both of the components of X574175 was lower in female mice, even though female mice were exposed to an average of 24% higher chemical due to their higher food consumption compared to males (Fig. 4). The mechanism(s) accounting for the observed high-dose nonlinearity are not understood but could include lower absorption from the GI tract, higher metabolic first-pass effect, more efficient elimination processes, or combination of all these factors resulting in lower serum levels of each component in the female mice. The effects noted in mice were opposite of those in rats,

where higher levels of both of the components were found in female than male rats (terminal serum data for female rats are not shown).

Effective and efficient collection of key pharmacokinetic values under actual conditions in which the toxicology information is being generated (e.g., diet, drinking water) provides the prospect of improving not only the specific design and interpretation of such studies but also the relevance to potential human risk. Knowledge of pharmacokinetic performance under actual test conditions may reveal important insights into potential relationships such as dose-dependency, mode (e.g., diet, bolus gavage, drinking water) and/or route of administration, and perhaps most importantly, blood/plasma concentrations observed at doses producing toxicity in animal studies as compared to concentrations resulting or estimated from real-world human exposures. These opportunities can be visualized from several of the data analyses in this report.

The data from the both 2,4-D and X574175 studies provide clear evidence of high-dose-dependent nonlinear pharmacokinetics (Tables 1, 2; Figs. 3, 4). The disproportionate increase in plasma 2,4-D concentrations at 100 mg/kg/day dietary treatment is important in that this is the LOEL dose in standard rat subchronic dietary studies (Charles et al., 1996) and is below the 150 mg/kg/day LOEL for 2,4-D in a chronic 1 year dietary neurotoxicity study (Mattsson et al., 1997). Thus, toxicity noted in rat dietary studies occurred only under conditions of nonlinear pharmacokinetic behavior, directly questioning the relevance of such high-dose toxicity findings in projecting potential risks associated with much lower worst-case human exposures to 2,4-D (e.g., estimated 0.3–6 μg/kg/day for commercial pesticide applicators; Yeary, 1996). The mechanism of the non-dose-proportional increase in plasma 2,4-D concentration is likely due to high-dose-dependent saturation of the renal active anion transport clearance mechanism, a mechanism also important for renal clearance of 2,4-D in humans (Timchalk, 2004). Unlike 2,4-D, the mechanisms accounting for the nonlinear plasma concentrations of the components of X574175 need further investigation. However, these data nonetheless provide valuable information for dose selection decisions in the design of future chronic rodent bioassays of this material.

Determination of plasma concentrations in standard dietary toxicity studies also allows for more effective comparison to toxicity studies routinely conducted by bolus oral gavage administration, for example, developmental toxicity bioassays. Administration of a single bolus dose of 2,4-D (5 mg/kg) to male F344 rats resulted in a plasma C_{max} of approximately 10 μg/ml 1 h after dosing and a C_{min} of 0.012 μg/ml within 12 h, an 806-fold difference between C_{max} and C_{min} (van Ravenzwaay et al., 2003). The same dose of 5 mg/kg/day administered in diet (this report) produced a C_{max} of only 0.72 μg/ml (14-fold lower) and C_{min} of 0.41 μg/ml (33-fold higher), affording a peak and trough plasma concentrations difference of only ~2-fold (Table 3). Importantly, the C_{max} after bolus dose was 14-fold higher than the dietary dose, although the difference between AUC values was only 1.6-fold (AUC after bolus dose = 21 μg h ml^{-1}; AUC after dietary exposure = 13 μg h ml^{-1}; van Ravenzwaay et al., 2003; Table 4). Such differences may be important considerations in comparisons of NOEL values derived from different modes of compound treatment.

The difference in variability between plasma C_{max} and C_{min} in animals given equivalent oral bolus or dietary doses of 2,4-D is largely due to the fact that the ingestion of a chemical provided through diet occurs throughout the day, causing the diurnal fluctuations within a relatively narrow range. In contrast, after a single bolus dose, most of the administered chemical is absorbed quickly from the GI tract producing a much higher C_{max} which is followed by a continuous and rapid decline in concentration resulting in a much larger difference between peak and trough values. This is particularly true for chemicals which have relatively short elimination half-lives.

Generation of kinetic data after single or multiple bolus doses for pharmaceutical drugs is in accordance with their use, however, the use of such data for environmental chemicals is justified only in the cases of accidental and/or incidental exposure scenarios. As is apparent with chlorpyrifos (Tables 1, 3 and 4), the kinetics of parent compound are entirely different for the bolus oral relative to dietary administration routes. No parent compound was detected after dietary exposure (this report) of 5 mg/kg/day, whereas circulating levels of parent compound were observed up to 6 h post-dosing in the blood of adult male F344 rats as well as in the blood and milk of Sprague–Dawley dams after a comparable bolus oral dose of 5 mg/kg (Mattsson et al., 2000; Timchalk et al., 2002). Parent compound was also detected in the blood of fetuses and pups of the dams bolus-dosed with chlorpyrifos (Mattsson et al., 2000). The C_{max} of the parent compound (30.4 ng/ml) in the blood of the male F344 rats receiving a bolus dose was reached within 3 h after dosing (Timchalk et al., 2002), which was in contrast to the dietary exposure at the same dose level where blood concentration remained below the detection limit of 0.3 ng/ml. The difference in the level of parent compound in blood circulation after dietary versus gavage administration is most probably due to high first-pass metabolism of chlorpyrifos following small but frequent intake via diet compared to bolus administration. These data indicate that the mode of dose administration (e.g., oral bolus vs. diet) may have important implications for expression of toxicity. By way of example, such phenomena may explain why the reproductive toxicity of dibutylphthalate in rats after dietary exposure was dramatically different from that following an equivalent (mg/kg/day) oral bolus dose (Patel et al., 2001).

Another potential value for determining plasma pharmacokinetic values in animal dietary toxicity studies is the

prospect of better relating the dose response information from these studies directly to the concentrations of chemicals and/or their metabolites increasingly being collected in human biomonitoring programs. Currently, human biomonitoring programs generate blood or urine concentration data in the absence of any or limited dose context to findings in toxicology studies. If current toxicity study designs were modified to include blood and urine analyses using approaches as suggested in this report, potentially more meaningful animal to human dose–exposure correlations could be developed (that is, human blood levels collected under conditions of real-world exposure could be compared to blood concentrations of chemicals in animal toxicity tests at both NOEL and LOEL response levels). Such comparisons might also provide future directions for more rationale selections of top and other dose levels of animal toxicity studies intended to explore potential human health risks of environmental chemicals.

In conclusion, this report describes diurnal variations in blood levels and elimination rates in subchronic/chronic feeding/drinking studies after reaching steady-state exposure levels and strategies for the estimation of systemically available daily doses of a chemical, utilizing a minimal number of blood samples. The best predictions of AUC-24 h were made when three time points were used, corresponding to C_{max}, a mid-morning sample, and C_{min}. Calculation of AUC-24 h from two samples (C_{max} and C_{min}) or one mid-morning sample afforded slightly larger variations. These data provide insight into systemic bioavailability as well as dose proportionalities. Coupled with estimates of C_{max} and C_{min}, collection of terminal blood sample from rats, routinely conducted 16–18 h after the removal of exposure, provides preliminary rough estimates of the blood/plasma half-life of a chemical for other PK studies. Determining the amount of chemicals and/or metabolite(s) in excreta may be useful as a biomarker in estimating the daily systemic exposure for sound risk assessment of environmental chemicals.

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