attributable to saturation of brain OAT1 clearance. However, the potential for high-dose-specific neurotoxicity associated with elevated brain concentrations of 2,4-D is dependent on such a sequential coupling of both kidney and brain OAT1 saturation that it would not be relevant to human risk assessment given the wide separation between doses required for saturation and actual human exposures.

The gender differences observed in dietary TK in this study are entirely consistent with sex-dependent differences in 2,4-D clearance mediated by OAT1 (Gorzinski *et al.*, 1987; Timchalk, 2004). Reyes *et al.* (1998) demonstrated that male rats have higher renal excretion rates for organic acids than females. This difference was hypothesized to be due to a testosterone-induced increase in the number of functional transporters available in the kidney for organic acid secretion, a finding consistent with the observation that adult male rats express higher levels of OAT1 than adult females (Buist *et al.*, 2002). These gender differences in OAT1 and organic acid transport are consistent with the current TK data, wherein female rats exhibited nonlinear TK at lower doses than males. The slower elimination of 2,4-D in females also was reported by van Ravenzwaay *et al.* (2003) after **PO** gavage dosing.

The 2,4-D life-stage-specific TK findings of this study also are consistent with the developmental ontogeny of OAT1. The dose-dependent clearance of 2,4-D in male and female pups was similar on PND 4, 21, and 28, but by PND 35, saturation of renal clearance was apparent in females at lower doses relative to males (Tables 1 and 4). Functionally, accumulation of the organic acid *p*-aminohippuric acid in rat kidney slices increased by approximately 2-fold between GD 20 fetuses and PND 6 pups, and approximately 3-fold between GD 20 fetuses and adults (Nakajima *et al.*, 2000). These results are consistent with the developmental expression of OAT1, where expression increased 4-fold between PND 5 and 35 in both male and female rats (Buist *et al.*, 2002). This developmental delay in achieving adult levels of OAT1 transport capacity, when coupled with increased dose (g/kg body weight) in pre- and early postweaning rats, likely contributed to the increased blood levels of 2,4-D at these early postnatal life stages. By PND 35, males express more OAT1 messenger RNA than females, and this difference in OAT1 expression becomes significant by PND 40 (Buist *et al.*, 2002). The timing of the gender-related difference in OAT1 expression is consistent with the appearance of gender-related differences in 2,4-D $AUCs_{24\,h}$ on PND 35.

The results of this extensive dietary and life-stage-dependent determination of 2,4-D TK also demonstrate that toxicity resulting from **PO** gavage dosing regimens potentially overestimates both hazard and risk potential relative to studies using equivalent daily dietary doses of 2,4-D. This potential is most clearly evidenced by comparing dietary male and female plasma C_{max} concentrations observed in this study (Supplementary Tables 6 and 7) to those reported in rats treated by **PO** gavage (van Ravenzwaay *et al.*, 2003). Plasma C_{max} concentrations were 0.73 and 1.29 µg/g, respectively, for male and female rats treated with approximately 5 mg/kg/day (100 ppm TD 28 and 29; Table 1) in the diet, whereas plasma C_{max} were elevated to 9.84 and 14.26 µg/g, respectively, for rats dosed at 5 mg/kg/day by **PO** gavage. The differences in plasma C_{max} were magnified to a greater extent as the daily doses approached or exceeded renal saturation. A daily dietary dose of 41 mg/kg/day (800 ppm, TD 28; Table 1) resulted in a plasma C_{max} of 10.14 µg/g in male rats compared with 189.8 µg/g in rats treated at the approximate equivalent dose of 50 mg/kg/day by **PO** gavage. A daily dietary dose of 52 mg/kg/day (800 ppm, TD 29; Table 1) in female rats produced a C_{max} of 48.58 µg/g versus 266.6 µg/g in females treated at the equivalent 50 mg/kg/day by **PO** gavage. 2,4-Dichlorophenoxyacetic acid is rapidly absorbed and exhibits nearly complete bioavailability by both **PO** gavage and dietary routes (Timchalk, 2004). Thus, the higher C_{max} values via **PO** gavage are related to rapid absorption of a bolus dose of 2,4-D, which more quickly saturates renal clearance. The slower dose rate achieved with the spreading of dietary exposures over the course of a day both prolongs the time to renal saturation for a given dose of 2,4-D as well as speeds up the time for recovery from saturation during noneating periods, resulting in lower C_{max} values via the dietary route. Because dietary exposure more closely represents the mode of exposure concern for the general human population, ie, pesticide residues in foods (Cooper *et al.*, 2006), these disparate plasma 2,4-D concentrations suggest that toxicity studies conducted by **PO** gavage likely overestimate toxicity potential in humans.

Although many of the toxicity studies used to support the registration of 2,4-D as a pesticide are indeed conducted by dietary administration, 2,4-D developmental toxicity studies in particular have routinely been performed using **PO** gavage dosing (Charles *et al.*, 2001). Finally, it is important to note that the dietary TK observed in parental rats treated up to 71 (males) and 95 (female) days in this study indicate that all of the reported systemic toxicity responses in adult rats, which have been used to establish regulatory health standards for 2,4-D (thyroid, adrenals, eye, ovaries/testes, and nervous system), with the exception of kidney, are associated with saturation of renal clearance (USEPA, 2005). In this study, only very slight kidney toxicity was seen in male rats at a subsaturating dose of 400 ppm (Table 2), whereas female kidney toxicity was only observed at the highly renal clearance saturating dose of 800 ppm. Although the mode of action of the sex-specific sensitivity of male rats to kidney toxicity is uncertain, it may be attributable to the higher level of expression of OAT1 and associated higher threshold for renal saturation in male relative to female rats, allowing for a higher delivered dose of 2,4-D to proximal tubule cells.

In summary, the example of 2,4-D illustrates how variations in TK over both dose and life stage can be used to define a scientifically sound and robust approach for dose selection in the design of an extended 1-generation reproduction study, including revealing systemic dose implications associated with significant changes in food intake occurring during periods

of rapid postnatal development in rats (Marty *et al.*, 2013). Importantly, the findings presented in this study have several broader implications for both improvements to future toxicity test design and for understanding the human risk relevance of previously reported high-dose-specific toxicity findings. These data are perhaps the first comprehensive examination of dietary dose- and life-stage-specific TK performance for a compound exhibiting dose-dependent saturation of metabolic clearance, and as such, illustrate how the KMD approach, which when coupled to human exposure information, significantly improves confidence in human risk extrapolation relative to conventional MTD-based study designs. Thus, for 2,4-D, application of the KMD approach confirmed that the highest toxicity test dose should be 2- to 4-fold lower than that suggested by an MTD approach, ultimately allowing for selection and/or interpretation of experimental doses more informative of real-world human exposures. This study findings are also yet another example demonstrating that mode of dose delivery is a potentially important factor impacting expression of toxicity, for example, **PO** gavage studies may overestimate toxicity potential for compounds subject to saturation of metabolic processes controlling toxicity and whose primary human **PO** exposures are through diet or drinking water. Of course, it is recognized that 2,4-D represents a relatively simple example of how saturation of a metabolic process(es) influences KMD evaluations in that 2,4-D does not undergo significant and potentially complicating toxification/detoxification metabolism, and its TK is largely influenced just by dose-dependent saturation of the OAT1 renal transporter in both animals and humans. Finally, this study has also shown that sex-dependent physiologic and/or metabolic factors also can be additional variables in KMD-based evaluations.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

ACKNOWLEDGMENTS

The authors would like to acknowledge Darlene Curell, Rebecca Drury, Kathy Gallagher, Barb Hall, Jane Lacher, Marie Lawson, Lisa McFadden, Beth Stieve, Amy Clark, Jennifer Staley, Barry Yano, and the histology and animal care staff of The Dow Chemical Company for their help in completing this study. The authors work for a company at which 2,4-D is produced or consult for the Industry Task Force II on 2,4-D Research Data.

REFERENCES

Alexander, B. H., Burns, C. J., Bartels, M. J., Acquavella, J. F., Mandel, J. S., Gustin, C., and Baker, B. (2007). 2,4-D Exposure in farm families: Results from the Farm Family Exposure Study. *Environ. Health Perspect.* **115,** 370–376.

Arnold, E. K., and Beasley, V. R. (1989). The pharmacokinetics of chlorinated phenoxy acid herbicides: A literature review. *Vet. Hum. Toxicol.* **31,** 121–125.

Barton, H. A., Pastoor, T. P., Baetcke, K., Chambers, J. E., Diliberto, J., Doerrer, N. G., Driver, J. H., Hastings, C. E., Iyengar, S., Krieger, R., *et al.* (2006). The acquisition and application of absorption, distribution, metabolism, and excretion (ADME) data in Agricultural Chemical Safety Assessments. *Crit. Rev. Toxicol.* **36,** 9–35.

Berndt, W. O., and Koschier, F. (1973). In vitro uptake of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) by renal cortical tissue of rabbits and rats. *Toxicol. Appl. Pharmacol.* **26,** 559–570.

Buist, S. C., Cherrington, N. J., Choudhuri, S., Hartley, D. P., and Klaassen, C. D. (2002). Gender-specific and developmental influences on the expression of rat organic anion transporters. *J. Pharmacol. Exp. Ther.* **301,** 145–151.

Bus, J. S., and Hammond, L. E. (2007). Regulatory process, toxicology, and public concerns with 2,4-D, where do we stand after two decades? *Crop Prot* **26,** 266–269.

Carmichael, N. G., Barton, H. A., Boobis, A. R., Cooper, R. L., Dellarco, V. L., Doerrer, N. G., Fenner-Crisp, P. A., Doe, J. E., Lamb, J. C. IV, and Pastoor, T. P. (2006). Agricultural Chemical Safety Assessment: A multisector approach to the modernization of human safety requirements. *Crit. Rev. Toxicol.* **36,** 1–7.

Charles, J. M., Bond, D. M., Jeffries, T. K., Yano, B. L., Stott, W. T., Johnson, K. A., Cunny, H. C., Wilson, R. D., and Bus, J. S. (1996a). Chronic dietary toxicity/oncogenicity studies on 2,4-dichlorophenoxyacetic acid in rodents. *Fundam. Appl. Toxicol.* **33,** 166–172.

Charles, J. M., Cunny, H. C., Wilson, R. D., and Bus, J. S. (1996b). Comparative subchronic studies on 2,4-dichlorophenoxyacetic acid, amine, and ester in rats. *Fundam. Appl. Toxicol.* **33,** 161–165.

Charles, J. M., Hanley, T. R., Jr, Wilson, R. D., van Ravenzwaay, B., and Bus, J. S. (2001). Developmental toxicity studies in rats and rabbits on 2,4-dichlorophenoxyacetic acid and its forms. *Toxicol. Sci.* **60,** 121–131.

Conolly, R. B., Beck, B. D., and Goodman, J. I. (1999). Stimulating research to improve the scientific basis of risk assessment. *Toxicol. Sci.* **49,** 1–4.

Cooper, R. L., Lamb, J. C., Barlow, S. M., Bentley, K., Brady, A. M., Doerrer, N. G., Eisenbrandt, D. L., Fenner-Crisp, P. A., Hines, R. N., Irvine, L. F., *et al.* (2006). A tiered approach to life stages testing for Agricultural Chemical Safety Assessment. *Crit. Rev. Toxicol.* **36,** 69–98.

Counts, J. L., and Goodman, J. I. (1995). Principles underlying dose selection for, and extrapolation from, the carcinogen bioassay: Dose influences mechanism. *Regul. Toxicol. Pharmacol.* **21,** 418–421.

Creton, S., Saghir, S. A., Bartels, M. J., Billington, R., Bus, J. S., Davies, W., Dent, M. P., Hawksworth, G. M., Parry, S., and Travis, K. Z. (2012). Use of toxicokinetics to support chemical evaluation: Informing high dose selection and study interpretation. *Regul. Toxicol. Pharmacol.* **62,** 241–247.

Doe, J. E., Boobis, A. R., Blacker, A., Dellarco, V., Doerrer, N. G., Franklin, C., Goodman, J. I., Kronenberg, J. M., Lewis, R., Mcconnell, E. E., *et al.* (2006). A tiered approach to systemic toxicity testing for Agricultural Chemical Safety Assessment. *Crit. Rev. Toxicol.* **36,** 37–68.

Garabrant, D. H., and Philbert, M. A. (2002). Review of 2,4-dichlorophenoxyacetic acid (2,4-D) epidemiology and toxicology. *Crit. Rev. Toxicol.* **32,** 233–257.

Gehring, P. J., and Betso, J. E. (1978). Phenoxy acids: Effects and fate in mammals. In *Chlorinated Phenoxy Acids and Their Dioxins* (C. Ramel, Ed.), Vol. **27**, pp. 122–133. Ecol. Bull. Oikos Editorial Office, Lund University, Sweden.

Gorzinski, S. J., Kociba, R. J., Campbell, R. A., Smith, F. A., Nolan, R. J., and Eisenbrandt, D. L. (1987). Acute, pharmacokinetic, and subchronic toxicological studies of 2,4-dichlorophenoxyacetic acid. *Fundam. Appl. Toxicol.* **9,** 423–435.

Griffin, R. J., Godfrey, V. B., Kim, Y. C., and Burka, L. T. (1997). Sex-dependent differences in the disposition of 2,4-dichlorophenoxyacetic acid in Sprague-Dawley rats, B6C3F1 mice, and Syrian hamsters. *Drug Metab. Dispos*. **25,** 1065–1071.

Hanley, T. R., Jr, and Watanabe, P. G. (1985). Measurement of solid feed consumption patterns in neonatal rats by 141Ce-radiolabeled microspheres. *Toxicol. Appl. Pharmacol*. **77,** 496–500.

Harris, S. A., and Solomon, K. R. (1992). Human exposure to 2,4-D following controlled activities on recently sprayed turf. *J. Environ. Sci. Health. B*. **27,** 9–22.

Hasegawa, M., Kusuhara, H., Endou, H., and Sugiyama, Y. (2003). Contribution of organic anion transporters to the renal uptake of anionic compounds and nucleoside derivatives in rat. *J. Pharmacol. Exp. Ther*. **305,** 1087–1097.

Haseman, J. K., and Hoel, D. G. (1974). Tables of Gehan's generalized Wilcoxon Test with fixed point censoring. *J. Statist. Comput. Simul*. **3,** 117–135.

Health Canada Pest Management Regulatory Agency (PMRA) (2008). Re-evaluation decision (2,4-dichlorophenoxy) acetic acid [2,4-D]. RVD2008-11. Pest Management Regulatory Agency, Health Canada, Ontario, Canada.

Hook, J. B., Bailie, M. D., Johnson, J. T., and Gehring, P. J. (1974). In vitro analysis of transport of 2,4,5-trichlorophenoxyacetic acid by rat and dog kidney. *Food Cosmet. Toxicol*. **12,** 209–218.

Kim, C. S., Gargas, M. L., and Andersen, M. E. (1994). Pharmacokinetic modeling of 2,4-dichlorophenoxyacetic acid (2,4-D) in rat and in rabbit brain following single dose administration. *Toxicol. Lett*. **74,** 189–201.

Kusuhara, H., and Sugiyama, Y. (2001a). Efflux transport systems for drugs at the blood-brain barrier and blood-cerebrospinal fluid barrier (Part 1). *Drug Discov. Today* **6,** 150–156.

Kusuhara, H., and Sugiyama, Y. (2001b). Efflux transport systems for drugs at the blood-brain barrier and blood-cerebrospinal fluid barrier (Part 2). *Drug Discov. Today* **6,** 206–212.

Marty, M. S., Neal, B. H., Zablotny, C. L., Yano, B. L., Andrus, A. K., Woolhiser, M. R., Boverhof, D. R., Saghir, S. A., Perala, A. W., Passage, J. K., *et al.* (2013). An F1–extended one generation reproductive toxicity study (EOGRTS) in CRL: CD(SD) rats with 2,4-dichlorophenoxyacetic acid (2,4-D). *Toxicol. Sci*. (Accepted).

McCoy, A. T., Bartels, M. J., Rick, D. L., and Saghir, S. A. (2012). TK Modeler version 1.0, a Microsoft® Excel®-based modeling software for the prediction of diurnal blood/plasma concentration for toxicokinetic use. *Regul. Toxicol. Pharmacol*. **63,** 333–343.

Morgan, M. K., Sheldon, L. S., Thomas, K. W., Egeghy, P. P., Croghan, C. W., Jones, P. A., Chuang, J. C., and Wilson, N. K. (2008). Adult and children's exposure to 2,4-D from multiple sources and pathways. *J. Expo. Sci. Environ. Epidemiol*. **18,** 486–494.

Munro, I. C., Caro, G. L., Orr, J. C., Sund, K. G., Wilson, R. M., Kennepohl, E., Lynch, B. S., Jablinske, M., and Lee, N. L. (1992). A comprehensive, integrated review and evaluation of the scientific evidence relating to the safety of the herbicide 2,4-D. *J. Am. Coll. Toxicol*. **11,** 559–647.

Nakajima, N., Sekine, T., Cha, S. H., Tojo, A., Hosoyamada, M., Kanai, Y., Yan, K., Awa, S., and Endou, H. (2000). Developmental changes in multispecific organic anion transporter 1 expression in the rat kidney. *Kidney Int*. **57,** 1608–1616.

Nozaki, Y., Kusuhara, H., Kondo, T., Hasegawa, M., Shiroyanagi, Y., Nakazawa, H., Okano, T., and Sugiyama, Y. (2007). Characterization of the uptake of organic anion transporter (OAT) 1 and OAT3 substrates by human kidney slices. *J. Pharmacol. Exp. Ther*. **321,** 362–369.

Organisation for Economic Co-operation and Development (OECD) (2011). OECD guideline for the testing of chemicals number 443: Extended one generation reproductive toxicity study. Accessed July 28, 2011.

Reyes, J. L., Meléndez, E., Alegría, A., and Jaramillo-Juárez, F. (1998). Influence of sex differences on the renal secretion of organic anions. *Endocrinology* **139,** 1581–1587.

Saghir, S. A., Bartels, M. J., Rick, D. L., McCoy, A. T., Rasoulpour, R. J., Ellis-Hutchings, R. G., Sue Marty, M., Terry, C., Bailey, J. P., Billington, R., and Bus, J. S. (2012). Assessment of diurnal systemic dose of agrochemicals in regulatory toxicity testing–an integrated approach without additional animal use. *Regul. Toxicol. Pharmacol*. **63,** 321–332.

Saghir, S. A., Mendrala, A. L., Bartels, M. J., Day, S. J., Hansen, S. C., Sushynski, J. M., and Bus, J. S. (2006). Strategies to assess systemic exposure of chemicals in subchronic/chronic diet and drinking water studies. *Toxicol. Appl. Pharmacol*. **211,** 245–260.

Slikker, W., Jr, Andersen, M. E., Bogdanffy, M. S., Bus, J. S., Cohen, S. D., Conolly, R. B., David, R. M., Doerrer, N. G., Dorman, D. C., Gaylor, D. W., *et al.* (2004a). Dose-dependent transitions in mechanisms of toxicity: Case studies. *Toxicol. Appl. Pharmacol*. **201,** 226–294.

Slikker, W., Jr, Andersen, M. E., Bogdanffy, M. S., Bus, J. S., Cohen, S. D., Conolly, R. B., David, R. M., Doerrer, N. G., Dorman, D. C., Gaylor, D. W., *et al.* (2004b). Dose-dependent transitions in mechanisms of toxicity. *Toxicol. Appl. Pharmacol*. **201,** 203–225.

Smith, F. A., Nolan, R. J., Hermann, E. A., and Ramsey, J. C. (1980). Toxicokinetics of 2,4-dichlorophenoxyacetic acid (2,4-D) in Fischer 344 rats. Technical report of the Dow Chemical Company.

Thomas, K. W., Dosemeci, M., Hoppin, J. A., Sheldon, L. S., Croghan, C. W., Gordon, S. M., Jones, M. L., Reynolds, S. J., Raymer, J. H., Akland, G. G., *et al.* (2010). Urinary biomarker, dermal, and air measurement results for 2,4-D and chlorpyrifos farm applicators in the Agricultural Health Study. *J. Expo. Sci. Environ. Epidemiol*. **20,** 119–134.

Timchalk, C. (2004). Comparative inter-species pharmacokinetics of phenoxyacetic acid herbicides and related organic acids. evidence that the dog is not a relevant species for evaluation of human health risk. *Toxicology* **200,** 1–19.

United States Environmental Protection Agency (USEPA) (2005). Reregistration eligibility decision for 2,4-D. EPA 738-R-05-002. June 2005.

United States Environmental Protection Agency (USEPA) (2011). 2,4-D: Petition for the establishment of a new formulation of 2,4-D choline on herbicide tolerant field corn containing the aryloxyalkanoate dioxygenase-1 (AAD-1) gene. Decision letter from the Office of Chemical Safety and Pollution Prevention U.S. Environmental Protection Agency, Registration No. 62719-AGO. Accessed October 27, 2011.

van Ravenzwaay, B., Hardwick, T. D., Needham, D., Pethen, S., and Lappin, G. J. (2003). Comparative metabolism of 2,4-dichlorophenoxyacetic acid (2,4-D) in rat and dog. *Xenobiotica*. **33,** 805–821.

Widdowson, E. M. (1976). Changes in the body and its organs during lactation: Nutritional implications. *Ciba Found. Symp*. **45,** 103–118.

Winer, B. J. (1971). Statistical Principles in Experimental Design (2nd Edition). McGraw-Hill, New York, pp. 208–209, 201–204.

Yeary, R. A. (1986). Urinary excretion of 2,4-D in commercial lawn specialists. *Appl. Ind. Hyg*. **3,** 119–121.

Zhang, X., Acevedo, S., Chao, Y., Chen, Z., Dinoff, T., Driver, J., Ross, J., Williams, R., and Krieger, R. (2011). Concurrent 2,4-D and triclopyr biomonitoring of backpack applicators, mixer/loader and field supervisor in forestry. *J. Environ. Sci. Health. B*. **46,** 281–293.