Use of Online Microdialysis Sampling to Determine the in Vivo Rate of Phenol Glucuronidation in Rainbow Trout

John W. Nichols, Alex D. Hoffman, Patrick N. Fitzsimmons, Gregory J. Lien, and Correne T. Jenson

U.S. Environmental Protection Agency, Office of Research and Development, National Health and Environmental Effects Research Laboratory, Midcontinent Ecology Division, Duluth, Minnesota

Received December 13, 2007; accepted April 16, 2008

ABSTRACT:

A quantitative microdialysis (MD) sampling method was used to study phenol (PH) glucuronidation in vivo in rainbow trout. The method employs internal calibrators to account for changes in MD probe performance (in vitro-to-in vivo and sample-to-sample) and yields data of high temporal resolution that are well suited for developing kinetic models. Initially, trout were dosed with phenyl glucuronide (PG) by intravascular infusion for 24 h and then depurated for 48 h. Measured concentrations of PG in blood were well described by a one-compartment clearance-volume model. Mass-balance calculations showed that 93% of infused PG was eliminated in urine during the depuration period. Peak concentrations of PG in urine averaged 3.4 times higher than those in blood, and the fitted PG clearance constant (15.7 ml/kg/h) was about 2.6 times higher than the reported glomerular filtration rate for trout. These findings confirm earlier work suggesting that PG is actively secreted by the trout kidney. In a second set of experiments, trout were exposed continuously to PH in water. In vivo rate constants for PH glucuronidation were estimated using a pair of linked (PH and PG) one-compartment clearance-volume models. Expressed on a whole-fish basis, the glucuronidation rate averaged 0.049/h, which was about 7% of the total rate of PH elimination. This study demonstrates the utility of quantitative MD sampling for kinetic studies of xenobiotic metabolism in fish.

Microdialysis (MD) is a well established in vivo technique that is used to sample or deliver chemicals in blood or interstitial water of selected tissues. The MD method employs a semipermeable membrane that is perfused on its inner surface with physiological saline. Chemicals diffuse across the membrane and are collected for analysis by HPLC or other techniques, often without the need for additional processing steps. “On-line” MD is characterized by direct transfer of dialysate samples to the analytical instrument, resulting in near real-time analysis of samples. MD sampling methods were initially developed to measure endogenous compounds in the CNS (Benveniste and Hüttemeier, 1990) but have since been used in a wide range of scientific disciplines, including studies of chemical kinetics and biotransformation. The use of MD in pharmacokinetic studies with mammals has been extensively reviewed (Stähle, 1992; Elmqquist and Sawchuk, 1997; Davies, 1999; Hansen et al., 1999; Verbeeck, 2000).

In a pioneering effort, McKim et al. (1993) used MD probes implanted in the dorsal aorta of rainbow trout to measure blood borne concentrations of phenol (PH) and its phase II metabolic product, phenyl glucuronide (PG), during continuous PH exposures. This method was then combined with periodic urinary sampling to characterize the renal elimination of PH, PG, and two additional PH metabolites, phenyl sulfate and hydroquinone (McKim et al., 1999). These studies showed that phenyl sulfate and PG are the principal products of PH metabolism in trout and that the trout kidney actively secretes these conjugates to urine. Both findings are consistent with results obtained in previous whole-animal studies of PH metabolism by fish (Layiwola and Linneker, 1981; Nagel, 1983; Nagel and Urich, 1983). More recently, Solem et al. (2003) implanted MD probes into trout liver tissue and used them as both delivery (PH) and sampling (metabolites) devices to study phase I PH metabolism. Phase I pathways in the liver were shown to catalyze the hydroxylation of PH to hydroquinone and, to a lesser extent, catechol.

The studies conducted by McKim and coworkers (McKim et al., 1993, 1999; Solem et al., 2003) demonstrated the utility of MD sampling for qualitative studies of fish metabolism. The calibration procedures used by these investigators were not adequate, however, to support kinetic model development. Specifically, these investigations were performed using MD probes that were calibrated in vitro using stirred solutions spiked with test compounds. Numerous studies have shown that in vitro probe performance may differ substantially from that of a probe inserted into a tissue or blood vessel (Bungay et al., 1990).

ABBREVIATIONS: MD, microdialysis; HPLC, high-performance liquid chromatography; PH, phenol; PG, phenyl glucuronide; PNPG, p-nitrophenyl glucuronide; UDPGA, uridine 5′-diphosphoglucuronic acid; DOC, dissolved organic carbon; ECFV, extracellular fluid volume; ISFV, interstitial fluid volume.
In the present study, we used a quantitative on-line MD sampling method to measure PH and PG in the bloodstream of trout (Nichols et al., 2008). Experiments were conducted to characterize the elimination of PG following intravascular infusion and its appearance during continuous waterborne exposures to PH. Two linked, one-compartment clearance-volume models were then used to estimate an apparent whole-body PH glucuronidation rate constant. The MD method employed in this effort utilizes internal calibration standards to correct for in vitro-to-in vivo differences in probe performance and sample-to-sample changes that occur in vivo over time. The resulting data sets possess high temporal resolution and are well suited for kinetic model development. This study demonstrates the utility of MD sampling methods for in vivo quantitative studies of fish metabolism.

Materials and Methods

Chemicals. PH, [14C]PH, PG, p-nitrophenyl glucuronic (PNPG), and uridine 5′-diphosphoglucuronic acid (UDPGA) were obtained from Sigma-Aldrich (St. Louis, MO). [14C]PG (99% pure, 11.3 μCi/μmol) was synthesized from [14C]PH and UDPGA using phenobarbital-induced rat liver microsomes. Microsomes (8–10 mg/ml) were obtained using procedures described by Preston and Allen (1980) and frozen at −80°C. Preliminary studies showed that the high concentration of glyceral (50%) in the microsomal suspension interfered with the synthesis. Prior to use, therefore, microsomes were diluted with 20 volumes of ice-cold Tris-HCl buffer (20 mM, pH 7.6), homogenized, and reconstituted with centrifugation at 100,000 g for 60 min (4°C). The microsomal pellet was then resuspended in 1 volume of Tris-HCl buffer. Incubations were conducted for 2 h at 37°C by adding MgCl2 (100 μl, 100 mM in Tris-HCl), UDPGA (100 μl, 100 mM in Tris-HCl), and [14C]PH (100 μl, 100 mM in Tris-HCl) to 500 μl of microsomes. An additional 100 μl of microsomes was added, and the reaction was continued for 1.5 h. [14C]PH was isolated and purified using anion exchange and C18 SPE columns. Purity and specific activity were determined by HPLC (see below) and liquid scintillation counting.

Animals. Rainbow trout (Oncorhynchus mykiss) weighing about 300 g were obtained from the U.S. Geological Service Fish and Wildlife Service Laboratory (La Crosse, WI) and grown to the size (600–1000 g) required for MD studies. The fish were fed a commercial trout chow (Silver Cup; Nelson and Sons Inc., Murray, UT) and maintained on a natural (Duluth, MN) photoperiod at 11 ± 1°C. Water used for fish holding and waterborne exposures to PH was obtained directly from Lake Superior and treated by sand filtration before use. Bile was obtained from the gall bladder at the end of the experiment using a 3-ml syringe and 22-gauge needle. The animals used in these studies were selected by the Care and Use Committee and conducted in accordance with principles developed since the previous sampling time. Samples were obtained hourly (Schmidt and Kimerle, 1981). Each sample consisted of urine that had accumulated over a period of time (±0.5 h) for that compound.

In vivo chemical exposures were conducted using transected fish confined to respirometer-metabolism chambers (McKim and Goeden, 1982). A latex oral mask was sewn to each fish’s mouth to separate inspired and expired water flows. A second membrane, located just posterior to the pectoral fins, limited the exposure to the fish’s head region. Urine was collected continuously through a PE-90 catheter that was implanted into the urinary bladder (Schmidt and Kimerle, 1981). Each sample consisted of urine that had accumulated since the previous sampling time. Samples were obtained hourly during the first 8 h of each experiment and less frequently thereafter. Experiments were terminated by exposing fish to an overdose of tricaine methane sulfonate (MS 222; Finquel7; Argent Laboratories, Redmond, WA) in water. Bile was obtained from the gall bladder at the end of the experiment using a 3-ml syringe and 22-gauge needle. The animals used in these studies were selected without regard to gender. Additional details specific to each experimental design are given below. All studies were approved by an institutional Animal Care and Use Committee and conducted in accordance with principles developed by the U.S. Government’s Interagency Animal Committee (Interagency Research Animal Committee, 1985).

MD Sampling Method. MD probes (20,000-Da cut-off; 0.5 × 10-mm membrane; CMA/Microdialysis, Solna, Sweden) equipped with fused silica inlet and outlet tubings (0.10-mm i.d.; Agilent Technologies, Palo Alto, CA) were implanted in the dorsal aorta of rainbow trout using the method described by McKim et al. (1993). Sample-to-sample calibration of probe performance was accomplished using [14C]PH and PNPG as internal calibration standards for PH and PG, respectively (Nichols et al., 2008). The probe perfusate was Cortland’s physiological saline (Wolf, 1963) flowing at a rate of 1 μl/min.

PG Infusion Studies. Four fish were infused with PG for 24 h through an indwelling intravascular cannula implanted in the ventral aorta (Kiceniuk and Jones, 1977). The purpose of these experiments was to characterize the kinetics of PG elimination in the absence of PH, which would have resulted in concurrent PG synthesis. The dosing solution was PG in Cortland’s saline. Measured concentrations for each fish ranged from 46.3 to 54.1 μmol/ml, and the flow rate was 1 μl/min. The infusion was then stopped, and fish were allowed to depurate PG for an additional 48 h.

In Vivo Glucuronidation of PH. Six fish were exposed to PH in water for 48 h with the goal of estimating whole-body rates of PH glucuronidation. Exposures were initiated by the addition of PH to a chemical mixing chamber supplied with Lake Superior water. Measured mean concentrations (n = 9) for each fish ranged from 30.1 to 43.5 μmol/ml. Water from the mixing chamber was then provided to tanks at a flow rate (500 ml/min) greater than their respiratory requirement.

An empirical equation (Arnott and Gobas, 2004; eq. 4 in the cited work) was used to estimate PH binding in Lake Superior water based on measured concentrations of DOC and particulate organic carbon. The results of this analysis suggested that >99% of the total PH concentration in the exposure water existed as the freely dissolved form.

Tissue Distribution of [14C]PG. The tissue distribution of [14C]PG was investigated by injecting 12 fish with a bolus dose in Cortland’s saline. The purpose of these studies was to provide a tissue-specific basis for interpreting fitted volumes of PG distribution from kinetic modeling efforts (see below). PG dosing was performed by fitting each fish with an indwelling dorsal aortic cannula (Garey, 1969). The injection volume was adjusted to provide a nominal dose of 41.6 μmol/kg (specific activity = 7.4 μCi/μmol after dilution of the concentrated stock with unlabeled PG). Four fish were then euthanized by anesthetic overdose with MS 222 at each of three sampling times (1, 4, and 24 h), and triplicate samples (approximately 1 g) of whole blood, plasma, bile, kidney, liver, and white muscle were obtained from each animal. [14C]PG concentrations were determined by wet tissue oxidation (Packard 307 Tri-Carb Oxidizer; PerkinElmer Life and Analytical Sciences, Meriden, CT) followed by liquid scintillation counting. This analysis assumed that all radioactivity was present as the parent radiolabeled material.

Analysis of MD Samples. A CMA/160 on-line injector (CMA/Microdialysis) was used to inject dialysate samples onto a Beckman 127 HPLC system equipped with a model 168 diode array detector ( Beckman Coulter, Inc., Fullerton, CA) and ISCO FOXY 2130 fraction collector (ISCO, Lincoln, NE). Samples were analyzed using an Alltech Hypersil BDS (C18)5 -ul, 100 mM in Tris-HCl), 305 nm, respectively. [14C]PH concentrations were determined by liquid scintillation counting. This analysis assumed that all radioactivity was present as the parent radiolabeled material.

Calculation of in Vivo Analyte Concentrations from MD Sampling Data. The free concentration of PH or PG in the blood of rainbow trout was calculated at each sampling time as (Nichols et al., 2008):

$$C_{	ext{blood}} = C_{	ext{OUTER}}R_{L_{	ext{in},	ext{VIVO}}}$$

where $R_{L_{	ext{in},	ext{VIVO}}}$ refers to the in vivo “relative loss” of the internal calibrator for that compound.
log-transformed elimination data. This model can be solved analytically by estimating $k_{10}$, first order clearance constant for PH uptake from water (liters per hour per kilogram); $C_{PG,U,BLOOD}$, unbound concentration of PG in blood (micromoles per liter); $V_{D,PG}$, apparent volume of PG distribution, referenced to $C_{PG,U,BLOOD}$ (liters per kilogram). B, one-compartment model for PH kinetics during a continuous waterborne exposure to PH. The model terms are: $k_{12}$, first order elimination rate constant (liters per hour); $C_{PH,U,BLOOD}$, unbound concentration of PH in blood (micromoles per liter); $V_{D,PH}$, apparent volume of PH distribution, referenced to $C_{PH,U,BLOOD}$ (liters per kilogram). C, linked, one-compartment models for PH and PG during a continuous waterborne exposure to PH. The compartment on the left is the same as that represented in B. The model terms are: $k_{10}$, first order elimination rate constant for PH production (equivalent to PH glucuronidation; liters per hour); $k_{12}$, first order ELPH elimination rate constant (liters per hour); $C_{PH,U,BLOOD}$, unbound concentration of PH in blood (micromoles per liter); $V_{D,PH}$, apparent volume of PH distribution, referenced to $C_{PH,U,BLOOD}$ (liters per kilogram); $C_{PG,U,BLOOD}$, unbound concentration of PG in blood (micromoles per liter); $V_{D,PG}$, apparent volume of PG distribution, referenced to $C_{PG,U,BLOOD}$ (liters per kilogram).

FIG. 2. A, one-compartment intravascular infusion model for PG kinetics in trout. The model terms are: $K_0$, rate of PG infusion (micromoles per hour per kilogram); $k_{10}$, first order PG elimination rate constant (liters per hour); $C_{PG,U,BLOOD}$, unbound concentration of PG in blood (micromoles per liter); $V_{D,PG}$, apparent volume of PG distribution, referenced to $C_{PG,U,BLOOD}$ (liters per kilogram). B, one-compartment model for PH kinetics during a continuous waterborne exposure to PH. The model terms are: $k_{12}$, first order clearance constant for PH uptake from water (liters per hour per kilogram); $C_{PH,U,BLOOD}$, total concentration of PH in inspired water (micromoles per liter); $k_{10}$, first order PH elimination rate constant (liters per hour); $C_{PH,U,BLOOD}$, unbound concentration of PH in blood (micromoles per liter); $V_{D,PH}$, apparent volume of PH distribution, referenced to $C_{PH,U,BLOOD}$ (liters per kilogram). C, linked, one-compartment models for PH and PG during a continuous waterborne exposure to PH. The compartment on the left is the same as that represented in B. The model terms are: $k_{12}$, first order rate constant for PH production (equivalent to PH glucuronidation; liters per hour); $k_{10}$, first order ELPH elimination rate constant (liters per hour); $C_{PH,U,BLOOD}$, unbound concentration of PH in blood (micromoles per liter); $V_{D,PH}$, apparent volume of PH distribution, referenced to $C_{PH,U,BLOOD}$ (liters per kilogram); $C_{PG,U,BLOOD}$, unbound concentration of PG in blood (micromoles per liter); $V_{D,PG}$, apparent volume of PG distribution, referenced to $C_{PG,U,BLOOD}$ (liters per kilogram).

exposures to PH (see below). Model solutions were obtained numerically using a commercial software package (ACSL TOX; Aegis Simulation, Inc., Huntsville, AL). Optimization routines within ACSL Math/Optimize were used to estimate specified model parameters.

A pair of linked, one-compartment models was developed to describe the kinetics of PH and PG during waterborne exposures to PH (Fig. 2, B and C). The mass-balance equation that describes the kinetics of free (unbound) PH ($C_{PH,U,BLOOD}$; micromoles per liter) in blood is:

$$dC_{PH,U,BLOOD}/dt = (k_{10}C_{PH,U,BLOOD}/V_{D,PH}) - k_{10}C_{PH,U,BLOOD}$$  \hspace{1cm} (4)

where $k_{10}$ (liters per hour per kilogram) is a first order clearance constant for PH uptake from water, $C_{PH,WATER}$ (micromoles per liter) is the concentration of PH in inspired water (assumed to be 100% free), $k_{10}$ (1/h) is a first order elimination rate constant, and $V_{D,PH}$ (liters per kilogram) is the apparent volume of PH distribution, referenced to $C_{PH,U,BLOOD}$. In this model, $k_{10}$ represents the sum of all first order processes that contribute to PH elimination, including but not limited to branchial elimination and biotransformation.

To estimate $k_{10}$ and $k_{12}$ it was necessary to adopt a fixed value for $V_{D,PH}$. An estimate of $V_{D,PH}$ was obtained by calculating a fish/blood partitioning coefficient (total chemical basis) and then dividing this value by the free fraction of PH in blood. Following Nichols et al. (2006), a fish/blood partitioning coefficient was calculated as the quotient of fish/water and blood/water partitioning values. Fish/water and blood/water values were calculated using an empirical equation that relates partitioning to tissue lipid and nonlipid organic matter content (Arnot and Gobas, 2004; eq. 3 in the cited work):

$$P_{fish\text{-}blood\text{-}water} = \mu K_{OW} + \nu_{NL} K_{OW} + \nu_{NL} R_{B}$$  \hspace{1cm} (5)

where $\mu$ (unitless) is the fractional lipid content, $\nu_{NL}$ (unitless) is the fractional content of nonlipid organic matter, $\nu_{NL}$ (unitless) is the fractional water content, and $R_{B}$ (unitless) is an empirically determined constant. The $\nu_{NL}$ assigned to whole fish (0.098) was based on the value given by Nichols et al. (1990) for adult rainbow trout. The $R_{B}$ of the fish was assumed to be 0.75. By difference,
Simulations were run to optimize model parameter values for PH (kg) to calculate the PG concentration in urine at each time point: at all bound in blood (Nichols et al., 2008). PG in urine is also likely to be bound in blood (Nichols et al., 2008). The empirical constant β was set equal to 0.035, as suggested by Arnot and Gobas (2004). The estimated log \( K_{OW} \) value for PH is 1.50 (Hansch et al., 1995), which equates to a \( K_{OW} \) value of 31.6.

The fish/water partitioning coefficient calculated using these parameter assignments is 4.01, whereas the blood/water value is 1.44. Dividing the former by the latter gives a fish/blood partitioning ratio of 2.78. Using a ultrafiltration method, Schmieder and Henry (1988) determined that 58% of PH in trout plasma existed as the unbound form. Similar binding values (50–61% free) were determined by Nichols et al. (2008) for whole blood using MD sampling methods. In the present modeling effort, we assumed an unbound fraction of 0.60 and divided this value into the fish/blood partitioning estimate. The resulting estimate of \( V_{D,PH} \) is 4.63 l/kg.

By linking the two models, the value of \( C_{PFU,BLOOD} \) at each time point becomes an input to the equation that describes the kinetics of free PG in blood (\( C_{PFU,BLOOD} \); micromoles per liter):

\[
dC_{PFU,BLOOD}/dt = (k_{10} V_{D,PG}) C_{PFU,BLOOD}/VL than PG in blood and urine can be compared directly, assuming that free and total concentrations within each sample matrix are the same.

The average urine/blood concentration ratio at the end of the 24-h dosing period was 3.4 ± 0.8 (mean ± S.E., \( n = 4 \)). Kinetic parameters for PG in blood were estimated by fitting eq. 3 to measured concentrations of PG (Fig. 1C, solid line; Table 1). An elimination mass balance was constructed by summing the mass of PG in urine and bile samples collected throughout each experiment (Table 2). On average, 93% of the infused PG was accounted for in urine and bile samples; of this, greater than 98% was contained in urine.

In Vivo Rate of PH Glucuronidation. Figure 3 shows representative data from a trout exposed continuously to PH in water. PH was taken up rapidly by fish and approached a steady state in blood within 10 h of initiating the exposure. In each experiment, the steady-state concentration of free PH in blood was close to the total concentration in the exposure water. Fitted uptake (\( k_{10} \)) and elimination (\( k_{10} \)) rate constants for PH in all six test animals are given in Table 3.

Measured concentrations of PG in blood and urine increased throughout each PH exposure and were far from steady state at 48 h (Fig. 3, B and C). Fitted kinetic parameters for PG are given in Table 4. The mean rate constant for phenol glucuronidation (\( k_{12} \)) was 0.49/h. PG clearance values (\( CL_{PG} \)) reported in Table 4 can be compared directly with those given in Table 1. Although individual values in Table 4 exhibit greater variability than those in Table 1, the means are essentially the same. In contrast, the mean \( V_{D,PG} \) determined for PH-exposed animals (from fish that provided both blood and urine) was nearly twice that determined in PG infusion studies.

Interpretation of Fitted Model Parameters. An effort was made to interpret fitted model parameters based on existing knowledge of chemical kinetics in fish and estimated volumes of different blood and tissue subcompartments. For example, the fitted uptake clearance constant for PH (i.e., \( k_{10} \)) was 2.78 l/h/kg (Table 3). Measured ventilation volumes for the fish exposed to PH averaged 15.0 l/h/kg, which is comparable with ventilation volumes reported in other studies with large trout (Nichols et al., 1990). Dividing \( k_{10} \) by the measured ventilation volume provides an estimate of PH extraction efficiency across the gills. Extraction efficiencies calculated in this manner averaged 20% (Table 3).

A mechanistic model of chemical uptake across fish gills was presented by Erickson and McKim (1990). When parameterized for rainbow trout, this model predicts that the branchial uptake efficiency for PH should be about 20% (assuming a log \( K_{OW} \) value of 1.50 and molecular weight of 94; calculations not shown). Therefore, the results of the present study provide additional support for the Erickson and McKim (1990) model.

Clearance constants for PG (\( CL_{PG} \)) were evaluated by comparison with the glomerular filtration rate in trout, estimated by McKim et al. (1999) to be about 6.1 ml/h/kg. Average values from Tables 1 and 4.
were about 2.6 and 2.4 times the estimated glomerular filtration rate, respectively, again suggesting that PG is actively secreted to urine. However, these clearance values comprise only a small (approximately 2%) percentage of total renal blood flow in trout, estimated by Nichols et al. (1990) to be about 0.86 l/h/kg (based on the sum of estimated arterial and portal blood flows).

Fitted volumes of distribution for PG (V₁₂PHG) were evaluated by comparison with reported whole-body extracellular fluid volumes (ECFVs) in trout (Table 5). The mean of fitted volumes from the PG infusion studies (0.206 l/kg) was 10 to 20% greater than reported ECFV values (which range from 0.173–0.193 l/kg), whereas the mean of values determined in waterborne exposures to PH (0.360 l/kg) was nearly double the highest reported ECFV. In either case, however, fitted volumes of distribution were substantially less than 0.75 l/kg, suggesting that PG tends to be excluded from intracellular water.

### Distribution of [¹⁴C]PG

Measured [¹⁴C]PG concentrations in fish were expressed as a set of plasma/blood and tissue/blood concentration ratios at 1, 4, and 24 h postinjection (Table 6). A predicted plasma/blood value was obtained by assuming that PG does not cross the red cell membrane. Barron et al. (1987) reported that the hematocrit of rainbow trout blood averages about 0.3. Therefore, plasma volume as a fraction of total blood volume equals 0.7, and the expected plasma/blood concentration ratio is 1/0.7, or 1.43.

Predicted tissue/blood concentration ratios for liver and white muscle were calculated using the fractional blood volume and ECFV reported for each tissue (Table 5). These calculations were based on the assumption that [¹⁴C]PG distributes rapidly between blood and tissue water but is unable to cross cell membranes. The amount of chemical in the tissue at any point in time, therefore, reflects the summed contributions of chemical in both the blood and interstitial fluid spaces. Tissue plasma volume was calculated as 0.7 times the fractional blood volume (assuming a hematocrit of 0.3). Tissue interstitial fluid volume (ISFV) was calculated in turn as the difference between ECFV and tissue plasma volume. For the liver, these calculations result in an ISFV of 0.045 l/kg. For muscle, the calculated ISFV is 0.046 l/kg. Algebraic expressions were then developed to calculate the expected [¹⁴C]PG concentration in tissue relative to that of blood by summing the volume-weighted contributions of each tissue subcompartment. For example, assuming that the concentration of [¹⁴C]PG in blood is 1.0 μM, the expected concentration in liver is 1.0 times the fractional liver blood volume, plus 1.43 times the ISFV. In this calculation, the concentration of [¹⁴C]PG in the ISFV (1.43) is set equal to that in plasma, determined from the previously calculated plasma/blood concentration ratio (see above). Summing these chemical contributions results in a predicted liver/blood concentration ratio of 0.21. A similar calculation results in a predicted muscle/blood concentration ratio of 0.08.

Observed plasma/blood concentration ratios for [¹⁴C]PG were essentially identical to the predicted value and did not change over time (Table 6). Muscle/blood concentration ratios did not differ from the predicted value at the first two sampling times (1 and 4 h postinjection) but increased at 24 h, suggesting that a small amount of [¹⁴C]PG had diffused into muscle cells. Both findings are consistent with the suggestion that PG distribution is largely restricted to extracellular water.

Observed liver/blood concentration ratios were higher than the predicted value at all three sampling times and increased over time. These differences were significant at 4 and 24 h. Existing data (i.e., kidney blood and extracellular fluid volumes) were insufficient to predict a kidney/plasma concentration ratio for [¹⁴C]PG. However, observed ratios greater than 1.0 suggest that [¹⁴C]PG is actively taken up by kidney tissue.

### Discussion

Most of the in vivo metabolism studies with fish have been conducted by dosing animals with a chemical and allowing them to depurate in clean water. The rate of metabolism is then calculated as the difference between the observed rate of elimination of parent chemical and the rate of elimination expected due to nonmetabolic pathways (Sijm and Opperhuizen, 1988; de Wolf et al., 1993; Fisk et al., 1998; Wong et al., 2002). Metabolism rates determined in this manner represent the sum of metabolic activity occurring in all tissues. These fitted rate constants provide no information, however, on the identity and fate of metabolic products. In addition, because

### Table 2

<table>
<thead>
<tr>
<th>Fish</th>
<th>Mass Infuseda</th>
<th>Σ Urineb</th>
<th>Bilec</th>
<th>cΣ Urine + c Bile/cMass Infused</th>
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<tr>
<td>1</td>
<td>73.29</td>
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<td>0.63</td>
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<td>3</td>
<td>79.02</td>
<td>73.03</td>
<td>0.96</td>
<td>0.84</td>
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<td>4</td>
<td>66.68</td>
<td>65.90</td>
<td>0.93</td>
<td>1.00</td>
</tr>
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</table>

[a] Stock concentration (micromoles per milliliter) × the infusion rate (milliliters per hour) × 24 h.  
[b] Mass, summed across all urine samples.  
[c] Mass, measured in the bile sample taken at test termination.

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Fig. 3. Representative data from a fish exposed to PH in water for 48 h. Solid lines, optimized model simulations for this animal. A, kinetics of PH accumulation in blood. B, kinetics of PG accumulation in blood. C, kinetics of PG elimination in urine.
animals can be sampled over time, but each animal can be sampled only once. Tissue, blood, and water samples may also present analysts with challenges commonly associated with traditional sample processing to inject directly onto an analytical instrument. Therefore, many of the challenges associated with extraction and analysis of test chemicals and their metabolic products.

In the present study, we employed a quantitative MD sampling method to collect in vivo data for PH and PG in rainbow trout. This information was used to develop clearance-volume models for each compound. By linking the two models, it was possible to estimate the in vivo rate of PH glucuronidation. Additional fitted model parameters were interpreted in the context of existing models of chemical uptake at fish gills, measured rates of glomerular filtration, and the volumes of blood and tissue subcompartments (e.g., ECFV, ISFV) to provide detailed descriptions of chemical distribution and elimination.

Unlike studies based on parent chemical kinetics, the MD sampling method provides in vivo kinetic data for both the parent compound and its metabolic products. Because there is no need to withdraw blood from the animal, the sampling interval becomes limited only by the analytical run time. Dialysate samples are generally clean enough to inject directly onto an analytical instrument. Therefore, many of the challenges commonly associated with traditional sample processing (e.g., incomplete recovery) are minimized or eliminated altogether.

The closed nature of the on-line injection system is particularly well suited for use with volatile compounds like PH. PH was selected as a model compound to demonstrate the MD method because the pathways for its metabolism in fish are well known and relatively simple (Layiwola and Linnecai, 1981; Nagel, 1983; Nagel and Urich, 1983; McKim et al., 1993, 1999; Solem et al., 2003). In addition, PH is historically one of the larger naturally occurring phenolic compounds in rainbow trout (Bradbury et al., 1993). The principal advantage of this approach is that it provides a direct estimate of metabolic rate for a specific metabolic pathway.

The main difficulty is the technical challenge of collecting high-quality kinetic data for a parent chemical and its metabolic products. Traditional blood sampling methods are limited with respect to the number of samples that can be collected. Tissues and/or whole animals can be sampled over time, but each animal can be sampled only once. Tissue, blood, and water samples may also present analytical challenges associated with extraction and analysis of test chemicals and their metabolic products.

### Table 3

Fitted model parameters for phenol from the aqueous phenol exposure study

<table>
<thead>
<tr>
<th>Fish</th>
<th>Weight (kg)</th>
<th>( k_{11} ) (l/h/kg)</th>
<th>( k_{10} ) (l/h)</th>
<th>Vent. Vol. (l/kg)</th>
<th>( k_{12}/\text{Vent. Vol.} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.006</td>
<td>2.32 (0.12)</td>
<td>0.53 (0.03)</td>
<td>18.55</td>
<td>0.13</td>
</tr>
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<td>6</td>
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<td>2.17 (0.10)</td>
<td>0.59 (0.03)</td>
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<td>0.15</td>
</tr>
<tr>
<td>7</td>
<td>0.761</td>
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<td>0.25</td>
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<td>8</td>
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<td>3.29 (0.35)</td>
<td>0.88 (0.09)</td>
<td>10.63</td>
<td>0.31</td>
</tr>
<tr>
<td>9</td>
<td>0.886</td>
<td>2.45 (0.18)</td>
<td>0.59 (0.04)</td>
<td>19.71</td>
<td>0.12</td>
</tr>
<tr>
<td>10</td>
<td>0.875</td>
<td>2.96 (0.17)</td>
<td>0.65 (0.04)</td>
<td>13.30</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>0.893 ± 0.033</td>
<td>2.78 ± 0.20</td>
<td>0.68 ± 0.05</td>
<td>15.01 ± 1.40</td>
<td>0.20 ± 0.03</td>
</tr>
</tbody>
</table>

**Vent. Vol.** = ventilation volume.

* *Average of measured values from the study.

### Table 4

Fitted model parameters for phenyl glucuronide from the aqueous phenol exposure study

<table>
<thead>
<tr>
<th>Fish</th>
<th>( k_{12} ) (l/h)</th>
<th>( k_{20} ) (l/h/kg)</th>
<th>( V_{D,PG} ) (l/kg)</th>
<th>( Cl_{PG} ) (ml/h/kg)</th>
<th>( k_{12}/h_{\text{Vol}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.024 (0.001)</td>
<td>0.024 (0.003)</td>
<td>0.36 (0)</td>
<td>8.5</td>
<td>0.05</td>
</tr>
<tr>
<td>6</td>
<td>0.037 (0.003)</td>
<td>0.051 (0.007)</td>
<td>0.36 (0)</td>
<td>18.5</td>
<td>0.06</td>
</tr>
<tr>
<td>7</td>
<td>0.048 (0.005)</td>
<td>0.038 (0.006)</td>
<td>0.41 (0.07)</td>
<td>15.7</td>
<td>0.06</td>
</tr>
<tr>
<td>8</td>
<td>0.066 (0.004)</td>
<td>0.026 (0.002)</td>
<td>0.40 (0.03)</td>
<td>10.4</td>
<td>0.08</td>
</tr>
<tr>
<td>9</td>
<td>0.059 (0.004)</td>
<td>0.066 (0.007)</td>
<td>0.36 (0)</td>
<td>23.6</td>
<td>0.10</td>
</tr>
<tr>
<td>10</td>
<td>0.061 (0.003)</td>
<td>0.040 (0.003)</td>
<td>0.27 (0.02)</td>
<td>11.1</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>0.049 ± 0.006</td>
<td>0.041 ± 0.006</td>
<td>0.36 ± 0.02</td>
<td>14.6 ± 2.1</td>
<td>0.07 ± 0.01</td>
</tr>
</tbody>
</table>

* *Average of measured values from the study.

### Table 5

Blood volume, ECFV, and ISFV in liver, white muscle, and whole-body tissues of rainbow trout

<table>
<thead>
<tr>
<th></th>
<th>Blood Volume</th>
<th>ECFV</th>
<th>ISFV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.145±</td>
<td>0.145±</td>
<td>0.045±</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.012±</td>
<td>0.054±</td>
<td>0.046±</td>
</tr>
<tr>
<td>Whole body</td>
<td>0.050±</td>
<td>0.173–0.193±</td>
<td>0.137–0.158±</td>
</tr>
</tbody>
</table>

* *From Stevens (1968).

### Table 6

Fitted model parameters for phenyl glucuronide from the aqueous phenol exposure study

<table>
<thead>
<tr>
<th>Fish</th>
<th>( k_{12} ) (l/h)</th>
<th>( k_{20} ) (l/h/kg)</th>
<th>( V_{D,PG} ) (l/kg)</th>
<th>( Cl_{PG} ) (ml/h/kg)</th>
<th>( k_{12}/h_{\text{Vol}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.024 (0.001)</td>
<td>0.024 (0.003)</td>
<td>0.36 (0)</td>
<td>8.5</td>
<td>0.05</td>
</tr>
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<td>0.051 (0.007)</td>
<td>0.36 (0)</td>
<td>18.5</td>
<td>0.06</td>
</tr>
<tr>
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<td>0.038 (0.006)</td>
<td>0.41 (0.07)</td>
<td>15.7</td>
<td>0.06</td>
</tr>
<tr>
<td>8</td>
<td>0.066 (0.004)</td>
<td>0.026 (0.002)</td>
<td>0.40 (0.03)</td>
<td>10.4</td>
<td>0.08</td>
</tr>
<tr>
<td>9</td>
<td>0.059 (0.004)</td>
<td>0.066 (0.007)</td>
<td>0.36 (0)</td>
<td>23.6</td>
<td>0.10</td>
</tr>
<tr>
<td>10</td>
<td>0.061 (0.003)</td>
<td>0.040 (0.003)</td>
<td>0.27 (0.02)</td>
<td>11.1</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>0.049 ± 0.006</td>
<td>0.041 ± 0.006</td>
<td>0.36 ± 0.02</td>
<td>14.6 ± 2.1</td>
<td>0.07 ± 0.01</td>
</tr>
</tbody>
</table>

* *Average of measured values from the study.

### Table 7

Fitted model parameters for phenyl glucuronide from the aqueous phenol exposure study

<table>
<thead>
<tr>
<th>Fish</th>
<th>( k_{12} ) (l/h)</th>
<th>( k_{20} ) (l/h/kg)</th>
<th>( V_{D,PG} ) (l/kg)</th>
<th>( Cl_{PG} ) (ml/h/kg)</th>
<th>( k_{12}/h_{\text{Vol}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.024 (0.001)</td>
<td>0.024 (0.003)</td>
<td>0.36 (0)</td>
<td>8.5</td>
<td>0.05</td>
</tr>
<tr>
<td>6</td>
<td>0.037 (0.003)</td>
<td>0.051 (0.007)</td>
<td>0.36 (0)</td>
<td>18.5</td>
<td>0.06</td>
</tr>
<tr>
<td>7</td>
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<td>0.038 (0.006)</td>
<td>0.41 (0.07)</td>
<td>15.7</td>
<td>0.06</td>
</tr>
<tr>
<td>8</td>
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<td>0.026 (0.002)</td>
<td>0.40 (0.03)</td>
<td>10.4</td>
<td>0.08</td>
</tr>
<tr>
<td>9</td>
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<td>0.36 (0)</td>
<td>23.6</td>
<td>0.10</td>
</tr>
<tr>
<td>10</td>
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<tr>
<td></td>
<td>0.049 ± 0.006</td>
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<td>0.36 ± 0.02</td>
<td>14.6 ± 2.1</td>
<td>0.07 ± 0.01</td>
</tr>
</tbody>
</table>
infusion studies were consistent with this suggestion, whereas those determined in PH exposures exceeded the estimated ECFV by a factor of almost 2. This apparent difference between \( V_{D,PG} \) values from the PG infusion and PH exposure studies cannot presently be explained.

Although it may be speculated that PG concentrations in the liver of PH-exposed fish were much higher than those in the infused animals (due to hepatic conversion of PH to PG), it seems unlikely that this alone could account for the observed difference in \( V_{D,PG} \). Alternatively, PH may have been metabolized by UGT isoforms distributed among extrahepatic tissues. Because PH diffuses easily across cell membranes, this metabolism could result in the formation of PG within tissues that do not accumulate the compound when it is injected into the bloodstream. A third possibility is that PH formed within the kidney is secreted directly to urine without entering the general circulation. Under these circumstances, the amount of PG in urine would reflect both renal secretion of PG formed in the kidney and secretion of PG circulating in the bloodstream. Because the liver does not account for this possibility, the pool of circulating PG available for renal secretion (i.e., \( V_{D,PG} \)) would have to be increased to achieve concordance between observed clearance of PG from blood and measured concentrations in urine.

Using the methods outlined in this study, it is not possible to discriminate between PG in urine that originates from hepatic and extrahepatic glucuronidation. Experimental approaches that could be used to address this question include the use of in vitro and/or in situ systems to independently measure rates of hepatic PH glucuronidation. This information could then be compared with those measured following the injection of \([^{14}C]PG\) and measured concentrations in urine.

Regardless of where glucuronidation occurs in trout, it appears that this metabolic pathway does not contribute substantially to PH elimination. When fitted glucuronidation rate constants for individual fish were divided by corresponding PH elimination rate constants (Table 4, last column), glucuronidation was found, on average, to account for only 7% of total PH elimination. Some PH is cleared as the parent chemical in urine and bile, although measured concentrations suggest that these elimination routes account for less than 2% of total clearance (data not shown). Additional PH is cleared by other metabolic pathways, including sulfation and hydroxylation (Layiwola and Linneweber, 1981; Nagel, 1983; Nagel and Urich, 1983; McKim et al., 1993, 1999; Solem et al., 2003); however, the primary means by which fish eliminate PH is likely to be loss of parent chemical directly across the gills.

All of the PH exposures conducted in this effort were performed at one waterborne PH concentration. It was not possible, therefore, to rigorously test the concentration dependence of pathways responsible for PH glucuronidation and renal elimination of PG (e.g., by normalizing data sets collected at more than one dose level to the tested concentration). The results of the modeling effort suggest, however, that both pathways exhibit first order kinetics over a wide range of concentrations. Thus, simple models based on the assumption of first order kinetics provided a good fit to the data at all time points. As noted previously, PG concentrations in blood during the depuration phase of PG infusion experiments exhibited a log-linear decline over time, again suggesting first order kinetics.

In summary, this study demonstrates the utility of quantitative MD sampling for kinetic studies of xenobiotic metabolism in fish and provides new information for an important model compound (PH) and its principal metabolic product (PG). The time and effort required to perform these types of experiments create a large research investment in each individual animal; however, the resulting data sets are extremely detailed. A relatively small number of animals are required, therefore, to characterize kinetic behaviors of interest. Additional work is needed to determine the practical applications of MD sampling for other compounds and metabolic pathways.

**Acknowledgments.** We thank Douglas Kuehl for synthesizing the \([^{14}C]phenyl\) glucuronide used in the intravascular infusion studies and Margaret James, Patricia Schmieder, and Daniel Villeneuve for constructive reviews of the manuscript.

**References**


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Address correspondence to: John W. Nichols, U.S. Environmental Protection Agency, Midcontinent Ecology Division, 6201 Congdon Boulevard, Duluth, MN 55804. E-mail: nichols.john@epa.gov