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

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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 bio-transformation. 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 urine 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 Linnevar, 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).

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ABBREVIATIONS: MD, microdialysis; HPLC, high-performance liquid chromatography; PH, phenol; PG, phenyl glucuronide; PNPG, p-nitrophenyl glucuronide; UDPGA, uridine 5’-diphosphogluconic 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, 11.38 μCi/μmol) was synthesized from [14C]PH and UDPGA using phenobarbital-induced rat liver microsomes. Microsomes (8–10 μg/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 resuspended in Tris and organic carbon (DOC), dissolved oxygen, 85 to 100% of saturation; dissolved organic carbon (DOC), and repelleted by 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]PG 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 (La Crosse, WI) and fed on a natural (Duluth, MN) photoperiod with 20 volumes of ice-cold Tris-HCl buffer (20 mM, pH 7.6), homogenized, and resuspended in Tris and organic carbon (DOC), dissolved oxygen, 85 to 100% of saturation; dissolved organic carbon (DOC), and repelleted by 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]PG 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.

In vivo chemical exposures were conducted using spinally 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 air. Water used for fish holding and waterborne exposures to PH was obtained directly from Lake Superior and treated by sand filtration before use. Water chemistry characteristics were: total hardness, 45 to 46 mg/l as CaCO3; alkalinity, 41 to 44 mg/l as CaCO3; pH 7.6 to 7.8; total ammonia, <1 mg/l; dissolved oxygen, 85 to 100% of saturation; dissolved organic carbon (DOC), 1.3 to 10−6 kg/l; particulate organic carbon, 1.0 × 10−5 kg/l (estimate, based on measured concentrations of DOC and organic carbon values).

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The free concentration of PH or PG in the blood of rainbow trout was calculated at each sampling time as (Nichols et al., 2008):

\[ \text{Free concentration} = \frac{\text{Concentration in probe}}{1 + \frac{1}{\text{Partition coefficient}}} \]

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 nCi/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 radio-labeled 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 Hypersyl BDS (C18) 5-μm, 2.1 × 150-mm column (Alltech Associates, Deerfield, IL) at a flow rate of 0.2 ml/min. Solvent A consisted of 9% acetonitrile, 25 mM ammonium acetate, and 0.2% acetic acid. Solvent B consisted of 20% acetonitrile, 100 mM ammonium acetate, and 0.2% acetic acid. Separation of analytes was achieved by using 100% A for 6.3 min and switching to 100% B for the duration of the run. PH, PG, and PNPG were quantified by UV absorbance at 270, 275, and 305 nm, respectively. [14C]PH concentrations were determined by liquid scintillation counting of fractions collected over a 5-min period encompassing the observed PH retention time.

Analysis of Urine, Water, and Bile. Urine, water, and bile samples were injected onto a second HPLC unit, configured in the same manner as that used for analysis of MD samples. When necessary, bile samples were diluted with deionized water (10- to 100-fold) to result in analyte concentrations that were within the range of analytical standards. Measured analyte concentrations in urine were plotted against the midpoint of the time interval over which samples were collected.

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):

\[ C_{\text{blood}} = \frac{C_{\text{outlet}} - RL\text{IN VIVO}}{RL\text{OUT VIVO}} \] where \(RL\text{IN VIVO}\) refers to the in vivo “relative loss” of the internal calibrator for that compound.
This model can be solved analytically by estimating \( C \), where \( \frac{V_{D,PG}}{D_{PG}} \), 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); \( k_{10} \), 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 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); \( C_{PG,U,BLOOD} \), 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).

The mass-balance equation that describes the kinetics of free (unbound) PH in blood is:

\[
dC_{PH,U,BLOOD}/dt = (k_{01}C_{PH,WATER}/V_{D,PH}) - k_{10}C_{PH,U,BLOOD}
\] (4)

where \( k_{01} \) (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 hour) 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 \( k_{01} \) and \( k_{10} \) 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):

\[
P_{fish/blood} = n_{L}K_{OW} + n_{NL}K_{OW} + P_{W}
\] (5)

where \( P_{fish/blood} \) (unitless) is the fractional lipiod content, \( n_{L}K_{OW} \) (unitless) is the fractional content of nonlipid organic matter, \( n_{NL}K_{OW} \) (unitless) is the fractional water content, and \( P_{W} \) (unitless) is an empirically determined constant. The \( P_{fish/blood} \) assigned to whole fish (0.098) was based on the value given by Nichols et al. (1990) for adult rainbow trout. The \( P_{fish/blood} \) of the fish was assumed to be 0.75. By difference,

Model-Based Evaluation of Data. Blood data from the PG infusion study were log-transformed to examine the kinetics of elimination. Log-transformed values from the depuration phase of these experiments declined in a linear manner (Fig. 1A) suggesting that a one-compartment model was appropriate to describe the kinetics of PG in blood (Fig. 2A). The input term \( (k_{c} \text{ mmol/h/kg}) \) for this model is the rate of PG infusion, whereas the output term is the product of a first order rate constant \( (k_{10}; \text{ liters per hour}) \), the free (unbound) PG concentration in blood \( (C_{PG,U,BLOOD}; \text{ micromoles per liter}) \), and an apparent volume of distribution \( (V_{D,PG}; \text{ liters per kilogram}) \), referenced to \( C_{PG,U,BLOOD} \). This model can be solved analytically by estimating \( k_{10} \) from the slope of log-transformed elimination data. PG clearance \( (C_{PG} \text{ per liter}) \) and \( V_{D,PG} \) can then be estimated from the relationship (Gibaldi and Perrier, 1982):

\[
V_{D,PG} = k_{c}(1 - e^{-k_{10}t})/C_{PG_{max}}
\] (2)

where \( C_{PG_{max}} \) is the maximum attained concentration in blood (micromoles per liter), \( T \) is the time at which the infusion is terminated, and \( C_{PG_{max}} \) is the product of \( k_{c} \) and \( V_{D,PG} \).

In the present study, however, we elected to fit the terms in this model by integrating the derivative form of the mass-balance equation:

\[
dC_{PG,U,BLOOD}/dt = (k_{c}V_{D,PG}) - k_{10}C_{PG,U,BLOOD}
\] (3)

An advantage of this approach is that parameter values are estimated by fitting model simulations to the entire data set. More importantly, this approach is consistent with that used to estimate the rate of PG formation in waterborne 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. 2B and C). The model terms are:

- \( k_{12} \): first order rate constant for PG production (equivalent to PG glucuronidation; liters per hour);
- \( k_{10} \): first order PG 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).

The model:

\[
\frac{dC_{PH,U,BLOOD}}{dt} = \left( k_{01}C_{PH,WATER}/V_{D,PH} \right) - k_{10}C_{PH,U,BLOOD}
\] (4)

where \( k_{01} \) (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 hour) 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 \( k_{01} \) and \( k_{10} \) 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):

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where \( P_{fish/blood} \) (unitless) is the fractional lipiod content, \( n_{L}K_{OW} \) (unitless) is the fractional content of nonlipid organic matter, \( n_{NL}K_{OW} \) (unitless) is the fractional water content, and \( P_{W} \) (unitless) is an empirically determined constant. The \( P_{fish/blood} \) assigned to whole fish (0.098) was based on the value given by Nichols et al. (1990) for adult rainbow trout. The \( P_{fish/blood} \) of the fish was assumed to be 0.75. By difference,
Simulations were run to optimize model parameter values for PH (kilogram) to calculate the PG concentration in urine at each time point: 

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 value 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\textsubscript{D,PH} is 4.63 l/kg.

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

\[
dC_{\text{PG,U,BLOOD}}/dt = (k_{11}V_{\text{D,PG}}C_{\text{PH,U,BLOOD}}/V_{\text{D,PG}}) - k_{20}C_{\text{PG,U,BLOOD}}
\]  

(6)

In this equation, \( k_{11} \) (liters per hour) is the PH glucuronidation rate constant. The second term in eq. 6 describes the elimination of PG, and the product of \( k_{20} \) and \( V_{\text{D,PG}} \) yields a clearance constant (CL\textsubscript{PG}; liters per hour per kilogram) that can be compared with the product of \( k_{10} \) and \( V_{\text{D,PG}} \) from eq. 3. Initial simulations were run to optimize model parameter values for PH (\( k_{11} \) and \( k_{10} \)). These fitted values were then used as inputs to the linked model for PH and PG. Importantly, this linkage of models requires that concentration terms be expressed on a molar basis, preserving 1:1 stoichiometry for PH disappearance and PG formation.

Finally, assuming that all of the PG cleared from blood is secreted to urine (see Results, PG Infusion Studies), we multiplied the second term in eq. 6 by \( V_{\text{D,PG}} \) and divided by the measured urine flow rate (liters per hour per kilogram) to calculate the PG concentration in urine at each time point: 

\[
C_{\text{PG,URINE}} = k_{20}C_{\text{PG,U,BLOOD}}/V_{\text{D,PG}} \text{ urine flow rate}
\]  

(7)

Unfortunately, urinary catheters failed in three of the six fish exposed to PH (nos. 5, 6, and 9). Therefore, our approach to modeling this data set was tailored to the outcome of each experiment. For the three fish that provided both blood and urine data (nos. 7, 8, and 10), it was possible to fit all three model parameters that define the kinetics of PG in blood (\( k_{12}, k_{20}, \) and \( V_{\text{D,PG}} \)). This was done by simultaneously optimizing simulations of measured blood and urine data. The average \( V_{\text{D,PG}} \) for these three animals was 0.36 l/kg. This mean distribution parameter was then used to model blood data for the other three animals, yielding fitted values for \( k_{20}, \) and \( V_{\text{D,PG}} \).

### Results

**PG Infusion Studies.** Representative data from a fish infused with PG are shown in Fig. 1. Previously, it was shown that PG is poorly if at all bound in blood (Nichols et al., 2008). PG in urine is also likely to be unbound, given the dilute nature of urine produced by freshwater-acclimated fish. Therefore, measured concentrations of 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 (\( k_{11} \)) 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\textsubscript{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 \( V_{\text{D,PG}} \) 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 15.01 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\textsubscript{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

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**TABLE 1**

**Fitted model parameters from the phenyl glucuronide infusion study**

<table>
<thead>
<tr>
<th>Fish</th>
<th>Weight (kg)</th>
<th>( K_a ) (( \mu \text{mol/kg} ))</th>
<th>( k_{10} ) (l/h)</th>
<th>( V_{\text{D,PG}} ) (l/kg)</th>
<th>CL\textsubscript{PG} (( \mu \text{mol/kg} \cdot \text{h} ))</th>
<th>CL\textsubscript{ULU} (( \mu \text{mol/kg} \cdot \text{h} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.968</td>
<td>3.09</td>
<td>0.062 (0.009)</td>
<td>0.254 (0.028)</td>
<td>15.6</td>
<td>196.2</td>
</tr>
<tr>
<td>2</td>
<td>1.035</td>
<td>2.97</td>
<td>0.067 (0.001)</td>
<td>0.194 (0.004)</td>
<td>13.0</td>
<td>228.5</td>
</tr>
<tr>
<td>3</td>
<td>1.339</td>
<td>2.43</td>
<td>0.077 (0.002)</td>
<td>0.197 (0.004)</td>
<td>15.1</td>
<td>160.2</td>
</tr>
<tr>
<td>4</td>
<td>1.153</td>
<td>2.41</td>
<td>0.108 (0.003)</td>
<td>0.178 (0.005)</td>
<td>19.2</td>
<td>125.2</td>
</tr>
<tr>
<td></td>
<td>1.124 ± 0.070</td>
<td>2.72 ± 0.15</td>
<td>0.078 ± 0.009</td>
<td>0.206 ± 0.014</td>
<td>15.7 ± 1.1</td>
<td>177.6 ± 19.3</td>
</tr>
</tbody>
</table>

* Calculated as \( k_{10}V_{\text{D,PG}} \times 1000.  
* Predicted steady-state concentration of phenyl glucuronide; calculated as \( K_a/k_{20}V_{\text{D,PG}} \).
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 ($V_{D,PG}$) 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 $[^{14}C]$PG.** Measured $[^{14}C]$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 $[^{14}C]$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 $[^{14}C]$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 $[^{14}C]$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 $[^{14}C]$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 $[^{14}C]$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 $[^{14}C]$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 $[^{14}C]$PG. However, observed ratios greater than 1.0 suggest that $[^{14}C]$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

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**TABLE 2**  
**Elimination mass balance for infused phenyl glucuronide**

Summary data are given as the mean ± S.E.

<table>
<thead>
<tr>
<th>Fish</th>
<th>Mass Infused$^a$</th>
<th>$\Sigma$ Urine$^b$</th>
<th>Bile$^c$</th>
<th>$\Sigma$ Urine + $\Sigma$ Bile/Mass Infused</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>73.29</td>
<td>65.10</td>
<td>Unavailable</td>
<td>0.89</td>
</tr>
<tr>
<td>2</td>
<td>73.77</td>
<td>73.03</td>
<td>0.63</td>
<td>1.00</td>
</tr>
<tr>
<td>3</td>
<td>79.02</td>
<td>65.68</td>
<td>0.96</td>
<td>0.84</td>
</tr>
<tr>
<td>4</td>
<td>66.68</td>
<td>65.90</td>
<td>0.93</td>
<td>1.00</td>
</tr>
</tbody>
</table>

$^a$ Stock concentration (micromoles per milliliter) $\times$ the infusion rate (milliliters per hour) $\times$ 24 h. 
$^b$ Mass, summed across all urine samples. 
$^c$ Mass, measured in the bile sample taken at test termination.
they are based on parent chemical kinetics, metabolism rates determined in this manner may be difficult to estimate when the rate of metabolism is low relative to that of nonmetabolic elimination pathways.

In a small number of studies, investigators have estimated whole-animal rates of metabolism by measuring the appearance of metabolic products in fish or the exposure water during a defined (typically continuous) exposure (Karara and Hayton, 1984; Barron et al., 1990; 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.

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 path-ways for its metabolism in fish are well known and relatively simple (Layiwola and Linneweber, 1981; Nagel, 1983; Nagel and Urich, 1983; McKim et al., 1993, 1999; Solem et al., 2003). In addition, PH is broadly representative of a large number of phenolic environmental contaminants including substituted phenols, alkylphenols, and hydroxylated products of polyaromatic hydrocarbon metabolism.

Based on the observed tissue distribution of injected $^{14}C$PG, we might have expected that the $V_{D,PG}$ would be close to but somewhat larger than the whole-body ECFV. $V_{D,PG}$ values determined in PG

### Table 3

<table>
<thead>
<tr>
<th>Fish</th>
<th>$k_{10}$</th>
<th>Vent. Vol.</th>
<th>$k_{10}$/Vent. Vol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.049</td>
<td>0.041</td>
<td>0.036</td>
</tr>
<tr>
<td>6</td>
<td>0.057</td>
<td>0.040</td>
<td>0.036</td>
</tr>
<tr>
<td>7</td>
<td>0.066</td>
<td>0.026</td>
<td>0.040</td>
</tr>
<tr>
<td>8</td>
<td>0.059</td>
<td>0.066</td>
<td>0.036</td>
</tr>
<tr>
<td>9</td>
<td>0.061</td>
<td>0.040</td>
<td>0.027</td>
</tr>
<tr>
<td>10</td>
<td>0.049</td>
<td>0.041</td>
<td>0.036</td>
</tr>
</tbody>
</table>

### Table 4

<table>
<thead>
<tr>
<th>Fish</th>
<th>$k_{12}$</th>
<th>$k_{20}$</th>
<th>$V_{D,PG}$</th>
<th>$Cl_{PG}$</th>
<th>$k_{2}/V_{10}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.024</td>
<td>0.024</td>
<td>0.036</td>
<td>8.5</td>
<td>0.05</td>
</tr>
<tr>
<td>6</td>
<td>0.037</td>
<td>0.051</td>
<td>0.036</td>
<td>18.5</td>
<td>0.06</td>
</tr>
<tr>
<td>7</td>
<td>0.048</td>
<td>0.038</td>
<td>0.041</td>
<td>15.7</td>
<td>0.06</td>
</tr>
<tr>
<td>8</td>
<td>0.066</td>
<td>0.026</td>
<td>0.040</td>
<td>10.4</td>
<td>0.08</td>
</tr>
<tr>
<td>9</td>
<td>0.059</td>
<td>0.066</td>
<td>0.036</td>
<td>23.6</td>
<td>0.10</td>
</tr>
<tr>
<td>10</td>
<td>0.061</td>
<td>0.040</td>
<td>0.027</td>
<td>11.1</td>
<td>0.09</td>
</tr>
</tbody>
</table>

### Table 5

<table>
<thead>
<tr>
<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>
</tr>
<tr>
<td>Muscle</td>
<td>0.012</td>
<td>0.054</td>
</tr>
<tr>
<td>Whole body</td>
<td>0.050</td>
<td>0.173</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 isofoms 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 PG 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 model 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 extrapolated to the whole animal and compared with fitted rates of glucuronidation, assuming that the liver is the primary site of this activity. It also may be possible 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 PH 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

Interagency Research Animal Committee (1985) U.S. Government Principles for Utilization and

| TABLE 6 |
| Distribution of \([^{14}C]\)PG to plasma, whole blood, and tissues, expressed as a set of concentration ratios 1, 4, and 24 h postinjection |

<table>
<thead>
<tr>
<th>[([^{14}C])PG Plasma/Blood or Tissue/Blood Concentration Ratio]</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Predicted</em></td>
</tr>
<tr>
<td>1 h</td>
</tr>
<tr>
<td>Plasma/blood</td>
</tr>
<tr>
<td>Muscle/blood</td>
</tr>
<tr>
<td>Liver/blood</td>
</tr>
<tr>
<td>Kidney/blood</td>
</tr>
</tbody>
</table>

* Significant differences from predicted values; two-tailed Student’s \( t \) test, \( \alpha = 0.05 \).
* Time course data are given as the mean ± S.E. of 3 (1 h) or 4 (4 and 24 h) calculated concentration ratios.
* For details on calculation of predicted values, see text.
Phenol glucuronidation in rainbow trout

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