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Measuring In Vitro Biotransformation Rates of Super Hydrophobic Chemicals in Rat Liver S9 Fractions Using Thin-Film Sorbent-Phase Dosing

Yung-Shan Lee,[†] S. Victoria Otton,[†] David A. Campbell,[‡] Margo M. Moore,[§] Chris J. Kennedy,[§] and Frank A.P.C. Gobas*[,†,§]

[†]School of Resource and Environmental Management, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6

[‡]Department of Statistics and Actuarial Science, Simon Fraser University, Surrey, British Columbia, Canada V3T 0A3

[§]Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6

Supporting Information

ABSTRACT: Methods for rapid and cost-effective assessment of the biotransformation potential of very hydrophobic and potentially bioaccumulative chemicals in mammals are urgently needed for the ongoing global evaluation of the environmental behavior of commercial chemicals. We developed and tested a novel solvent-free, thin-film sorbent-phase in vitro dosing system to measure the in vitro biotransformation rates of very hydrophobic chemicals in male Sprague–Dawley rat liver S9 homogenates and compared the rates to those measured by conventional solvent-delivery dosing. The thin-film sorbent-phase dosing system using ethylene vinyl acetate coated vials was developed to eliminate the incomplete dissolution of very hydrophobic substances in largely aqueous liver homogenates, to determine biotransformation rates at low substrate concentrations, to measure the unbound fraction of substrate in solution, and to simplify chemical analysis by avoiding the difficult extraction of test chemicals from complex biological matrices. Biotransformation rates using sorbent-phase dosing were 2-fold greater than those measured using solvent-delivery dosing. Unbound concentrations of very hydrophobic test chemicals were found to decline with increasing S9 and protein concentrations, causing measured biotransformation rates to be independent of S9 or protein concentrations. The results emphasize the importance of specifying both protein content and unbound substrate fraction in the measurement and reporting of in vitro biotransformation rates of very hydrophobic substances, which can be achieved in a thin-film sorbent-phase dosing system.

INTRODUCTION

National and international regulatory programs, including the Canadian Environmental Protection Act (CEPA), the U.S. Toxic Substances Control Act (TSCA), the E.U. Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), and the UNEP Stockholm Convention on Persistent Organic Pollutants use the bioconcentration factor (BCF), bioaccumulation factor (BAF), or the logarithm of octanol–water partition coefficient (log K_{OW}) to assess the bioaccumulative behavior of commercial chemicals in food-webs.[1] However, since the BCF and BAF are not available for the great majority of commercial chemicals, the assessment of bioaccumulation often relies on the application of BCF and BAF bioaccumulation models, Quantitative Structure–Activity Relationships (QSARs) or the K_{OW} criterion (log K_{OW} > 5).[1,2] A key limitation of these assessment techniques is that they are poorly equipped or unable to estimate the rate of biotransformation of the chemical.[3] Underestimation of biotransformation rates may cause many chemicals to be mis-classified as bioaccumulative when they are not. To date, standardized protocols for determining biotransformation rates of chemicals do not exist. However, there is a growing need to develop methods for determining the rates of biotransformation of chemicals especially for very hydrophobic (log K_{OW} > 5) and poorly volatile (log K_{OA} > 6) chemicals that have a high bioaccumulation potential. Chemicals with a relatively low K_{OW} and/or K_{OA} are quickly eliminated in most organisms and typically do not biomagnify even if they are not subject to biotransformation.[4] However, chemicals of high K_{OW} and high K_{OA} are very slowly eliminated and even low rates of biotransformation can dominate the overall depuration rate of the chemical and determine whether or not the substance will biomagnify. For that reason, several authors have advocated for the development of methods for determining biotransformation rates that minimize animal testing, reduce costs, speed up the chemical evaluation process and use animal models other than fish.[3,5]

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The application of in vitro assays and in vitro-to-in vivo extrapolation (IVIVE) of biotransformation rates has been proposed as a potential solution.[3,5−12] This method has been used extensively in the pharmaceutical field to assess metabolic clearance rates of drugs.[13−17] In-vitro studies involving freshly isolated hepatocytes from fish[7−9] and rats,[7] fish liver microsomes[8,10] and fish liver S9 fractions,[9−11] perfused fish liver preparations[12] and models by Cowan-Ellsberry et al.[9] demonstrate that these methods may also be useful for determining the biotransformation rate of potentially bioaccumulative chemicals. However, the exceptionally high hydrophobicity of potentially bioaccumulative substances may limit the applicability of tests designed for relatively water-soluble pharmaceutical drugs. One limitation concerns the dissolution of extremely hydrophobic substances in a largely aqueous medium (e.g., liver cell suspensions or tissue homogenates). The "solvent-delivery" or "spiking" procedure used for this purpose can lead to incomplete dissolution of the test chemicals in the assay medium[18−21] causing underestimation of the biotransformation rate constant. A second limitation is the introduction of a spiking solvent in the assay which can inactivate metabolic enzymes or may cause competitive inhibition between the spiking solvent (at high concentration) and the test chemical (at low concentrations).[22−24] A third limitation concerns the applicability of the dosing regime of current in vitro bioassays to environmental exposures. Solvent-delivery methods for pharmaceutical drugs mimic typical oral drug administration conditions where organisms are exposed to high initial concentrations after oral administration. In contrast, exposures to environmental contaminants typically involve low concentrations over a prolonged period. Exposure conditions can affect reaction order and rate. For example, high initial concentrations in in vitro bioassays using solvent-delivery procedures can lead to enzyme saturation (if the substrate concentration exceeds the Michaelis−Menten constant), thereby yielding lower substrate biotransformation rate constants than would be achieved at much lower substrate concentrations. A fourth limitation is that the extrapolation of in vitro to in vivo biotransformation rates requires knowledge of the unbound chemical fraction in the incubation medium, which is not determined in the solvent delivery method and therefore requires additional experimentation or computational modeling.[9−11] Finally, the application of solvent-delivery methods for the measurement of biotransformation rates of large numbers of chemicals can pose analytical challenges associated with the extraction, separation and analysis of test chemicals in complex biological media such as liver S9, microsomes, or hepatocytes.

A solvent-free dosing technique has the potential to overcome these problems. Previous studies have shown that hydrophobic test chemicals loaded into solid sorbent phases such as poly(dimethylsiloxane) (PDMS) or ethylene vinyl acetate (EVA) can deliver a steady concentration of dissolved chemical in aqueous media.[21,25−30] Sorbent-phase dosing may be useful for measuring biotransformation rates of very hydrophobic chemicals with bioaccumulation potential because (i) it eliminates the problem of incomplete dissolution of very hydrophobic substances in aqueous tissue homogenates, (ii) it avoids the introduction of solvents into the incubation medium, (iii) it determines biotransformation rates at very low substrate concentrations, (iv) it measures the unbound fraction of substrate in solution, and (iv) it avoids chemical extraction from complex biological matrices if the chemical concentration in the sorbent phase is used for the determination of the biotransformation rate.

The objective of this study was to develop a thin-film sorbent-phase dosing system for measuring in vitro biotransformation rates of very hydrophobic chemicals by a rat liver S9 fraction, to compare the performance of this method to that of a conventional solvent dosing system and to investigate the role of the unbound substrate fraction and enzyme concentration on the in vitro biotransformation rate.

THEORY

Sorbent-Phase Dosing. Thin-film sorbent-phase dosing involves the diffusive delivery of the test chemical from a thin film of solid sorbent material such as ethylene vinyl acetate (EVA) into a largely aqueous medium containing metabolic enzymes such as an S9 liver homogenate, liver microsomes or a suspension of hepatocytes. The exchange of the test chemical between the sorbent phase and the medium can be described by a two-compartment mass-transfer model (Figure 1):

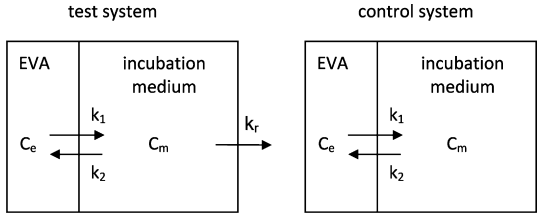

Figure 1. Two-compartment model of the thin-film sorbent-phase dosing system illustrating the chemical substrate concentration in the thin film sorbent (C_e) and in the incubation medium (C_m), the film to medium transfer rate constant k_1, the medium to film transfer rate constant k_2 and the biotransformation rate constant k_r.

$$\frac{dC_e}{dt} = k_2\frac{V_m}{V_e}C_m - k_1C_e \quad (1)$$

$$\frac{dC_m}{dt} = k_1\frac{V_e}{V_m}C_e - (k_2 + k_r)C_m \quad (2)$$

where C_e and C_m are the concentrations (mol/m^3) of the chemical in the EVA thin film and the incubation medium containing liver homogenate, respectively; V_e and V_m are the volumes of the EVA thin film and the incubation medium (m^3), respectively; k_1 and k_2 are the mass-transfer rate constants (min^{-1}) describing the transfer of the chemical from the thin film to the incubation medium (k_1) and from the medium back to the thin film (k_2), respectively; and k_r is the in vitro biotransformation rate constant (min^{-1}). Equation 1 describes the chemical exchange between the sorbent phase and the incubation medium. Equation 2 describes the role of biotransformation in the incubation medium.

Figure 2 illustrates that the biotransformation rate constant k_r can be determined by measuring the time course of the parent (i.e., unmetabolized) test chemical concentration in the sorbent phase (a) and the incubation medium (b) during a test (using a metabolically active medium) and a negative control (using an inactive medium that is, k_r is zero). Inactive incubation medium can be prepared by heat denaturation of the enzymes, the exclusion of cofactors for the enzymatic reaction from the incubation medium or the addition of inhibitors. The rate constants k_1 and k_2 are determined in the control experiments by measuring the

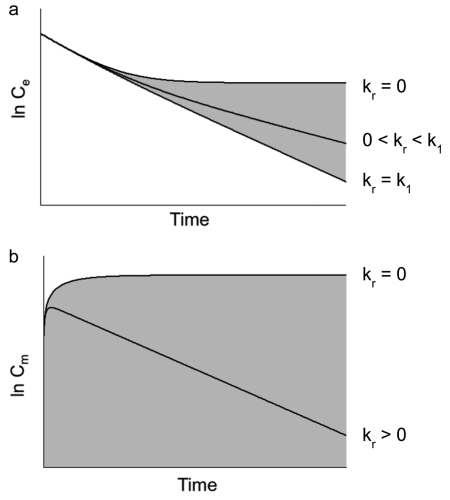

Figure 2. Diagrams illustrating the theoretical time course of the chemical concentration in the thin film sorbent phase (a) and the incubation medium (b) if the biotransformation rate constant $k_r = 0$ (control) and $k_r > 0$ (test). The shading illustrates the approximate range of k_r values that can be determined when measuring concentrations in the sorbent phase (a) and in the incubation medium (b).

time course of the test chemical concentration in either the sorbent phase or the incubation medium or both media. The biotransformation rate constant k_r, is determined by comparing the concentration time course in either the sorbent or incubation medium of the active system (i.e., test) to the corresponding concentration time course in the inactive system (i.e., control).

Figure 2 also illustrates the theoretical working range of thin film sorbent phase dosing. When measuring concentration time course in the sorbent phase (a), k_r values ranging from the detection limit (i.e., no statistically significant differences between the sorbent concentration time courses in the test (active medium) and control (inactive medium)) to approximately k_1 (i.e., the film to medium chemical delivery rate constant), which is determined in the control experiment. Very high biotransformation rates (i.e., $k_r > k_1$) may therefore not be measurable as temporal concentration changes in the sorbent phase because the sorbent to medium delivery rate is the rate controlling step in the biotransformation rate measurement. Such very high k_r values should be reported as values greater than k_1. The value of k_1 is determined in the control experiment and may be increased by adding stirring techniques to the method. For the classification of chemicals for bioaccumulation capacity, it is likely that a minimum in vitro biotransformation rate constant for nonbioaccumulative substances can be defined. If this in vitro biotransformation rate constant is less than k_1, observations indicating that $k_r > k_1$ may be sufficient for chemical classification. When measuring the concentration time course in the incubation medium (b), all k_r values exceeding the lower detection limit (i.e., no statistically significant differences between the incubation medium concentration time courses in the test (active medium) and control (inactive medium) can theoretically be measured. Very fast biotransformation reactions, including those for which $k_r > k_1$, can also be measured but may produce low concentrations in the incubation medium that do not meet the analytical detection limit. However, the measurement of the reaction rate in the incubation medium is not limited by the sorbent-to-medium delivery rate.

In Vitro-to-In Vivo Extrapolation (IVIVE). Several studies have shown that nonspecific binding can affect the extrapolation of in vitro biotransformation rates to in vivo rates.[31−34] This phenomenon is of particular importance to very hydrophobic chemicals with a high bioaccumulation potential because of the extremely high affinities of these chemicals for lipids, proteins and other biomolecules in biota. Therefore the rate of biotransformation in a biological medium (dC_m/dt) is often expressed in terms of the unbound or freely dissolved chemical concentration C_{fd}, which is related to the chemical concentrations in the reacting medium C_m by the fraction of unbound or freely dissolved chemical in the medium f_{fd}:

$$\frac{-dC_m}{dt} = \frac{V_{max}}{K_M + C_{fd}} C_{fd} = k_r^* C_{fd} = k_r^* f_{fd} C_m = k_r C_m \quad (3)$$

where V_{max} is the maximum velocity of the biotransformation reaction ($mol \cdot m^{-3} \cdot min^{-1}$), K_M is the Michaelis−Menten constant (mol/m^3), k_r^* is the intrinsic in vitro biotransformation rate constant of the unbound test chemical (min^{-1}) and k_r is the apparent in vitro biotransformation rate constant of the test chemical (min^{-1}). At low substrate concentrations (i.e., $C_{fd} \ll K_M$), the apparent biotransformation rate constant k_r follows first order kinetics and is the product of the intrinsic in vitro biotransformation rate constant k_r^* and the fraction of freely dissolved (or unbound) test chemical in the incubation medium, that is,

$$k_r = \frac{V_{max}}{K_M} f_{fd} = k_r^* \cdot f_{fd} \quad (4)$$

The unbound fraction f_{fd} is often difficult to determine experimentally for very hydrophobic chemicals, but in a sorbent-phase dosing experiment it can be derived from the chemical concentrations in the film (C_e) and the medium (C_m) in the control experiment (using an enzymatically inactive medium) at steady-state as $C_e/(K_{ew} \cdot C_m)$ where K_{ew} is the thin film to water partition coefficient of the chemical, which can be determined experimentally or through correlations with the octanol−water partition coefficient.[35] Theoretically, f_{fd} can be expressed as

$$f_{fd} = \frac{1}{1 + \sum_{i=1}^{n} \left(\frac{V_i}{V_w} \cdot K_{iw} \right)} \quad (5)$$

where V_i/V_w is the relative volume (m^3) of nonaqueous medium constituent i (e.g., protein or lipid) to water, n is all relevant nonaqueous medium constituents that can bind the enzyme substrate other than water and K_{iw} is the medium constituent i to water partition coefficient (unitless). This expression is similar to the equation used for calculating unbound fraction reported by Jones and Houston.[16] Substituting eq 5 in eq 4 shows that for relatively water-soluble substances which have a low K_{iw}, f_{fd} approaches 1 and the intrinsic biotransformation rate constant k_r^* approaches k_r. An increase in protein content in the incubation (e.g., a higher concentration of S9) can therefore be expected to increase V_{max}, k_r^* and k_r. This makes it necessary to normalize k_r to the protein content in the incubation medium when comparing in vitro clearance rates among different experiments and when extrapolating an in vitro biotransformation rate to an in vivo rate. For very hydrophobic substances for which $\Sigma(V_i \cdot K_{iw})/V_w \gg 1$ (i.e., the great majority of the chemical in the reaction medium is

bound), an increase in protein content (e.g., a more concentrated S9 medium) will increase V_{max} while reducing f_{fd}. For these substances, protein normalization of k_r can produce widely varying determinations of the biotransformation rate which are difficult to extrapolate to in vivo rates unless the fraction of unbound chemical in the test is taken into account. When extrapolating the experimentally observed apparent biotransformation rate constant to an in vivo biotransformation rate constant in a liver organ, it is important to account for differences in f_{fd} between the test system and the actual liver as well as the differences in protein concentrations in the test system and in the actual liver.

MATERIALS AND METHODS

Descriptions of the chemical substances used; the preparation of liver S9 homogenates and conditions for analysis by gas chromatography–mass spectrometry (GC/MS) in this study are included in the Supporting Information.

Thin Film Preparation. A 0.64 g/L EVA solution was prepared by dissolving EVA beads in dichloromethane. This solution was spiked with one of three test chemicals, that is, chrysene (log K_{OW} = 5.73), benzo[a]pyrene (log K_{OW} = 5.97) and PCB 153 (log K_{OW} = 7.50), to final concentrations of 2.74, 5.05, and 8.66 μg/mL, respectively. Thin films of EVA containing the test chemicals were formed on the interior surface of 2 mL silanized amber glass vials (Agilent, Mississauga, ON) by adding 25 μL of the EVA solution and rolling the vials slowly to evaporate the solvent. Each vial contained 0.016 μL (16 μg) of EVA, producing a film with an average thickness of 0.02 μm.[36]

Incubation Conditions of Sorbent-Phase Dosing System. The reactions were started by adding 0.5 mL incubation mixture (preincubated at 37 °C for 5 min) to the EVA-coated vials. The maximum possible concentrations of chrysene, benzo[a]pyrene, and PCB 153 in the incubation medium were 0.6, 1.0, and 1.0 μM, respectively, assuming that 100% of the chemicals were delivered to the incubation medium. The incubation mixture consisted of 0.2 mL phosphate buffer (0.2 M, pH 7.4), 0.1 mL KCl (1.15% w/v), 0.1 mL NADPH generating system (including 1.6 μmol NADP, 16 μmol glucose-6-phosphate, 1.6 units glucose-6-phosphate dehydrogenase following Van et al.[37] and 4 μmol $MgCl_2$ prepared in phosphate buffer), and 0.1 mL male Sprague–Dawley rat liver S9 (containing approximately 6 mg S9 protein). Incubations were conducted at 37 °C in a water bath (Grant OLS200, Cambridge, UK) that rolled the vials horizontally at 80 rpm to optimize contact between the incubation medium and the EVA thin film. Vials were capped with PTFE lined screw caps during the incubation to prevent evaporative losses of the test chemicals. At 2.5, 5, 10, 15, 30, 45, 60, 75, 90, 105, and 120 min, the reactions were stopped by quickly transferring 0.4 mL of incubation medium into 1 mL ice-cold hexane (for later extraction and analysis) and by removing the remaining medium from the EVA coated vials. The EVA thin films were then rinsed twice with 0.4 mL of deionized water and 1 mL hexane was added to the vials to extract chemicals from the films.

Three independent experiments were conducted to determine the average in vitro biotransformation rates of the test chemicals. In each experiment, a test system using the incubation conditions described above were run in parallel with two controls: a "no-cofactors control" in which the NADPH generating system was omitted from the incubation mixture, and a "heat-treated control" which used heat treatment (60 °C for 10 min) for enzyme inactivation. In each experiment, two vials without incubation medium were also incubated to determine the initial concentration of the test chemicals in the EVA thin films (C_e at t = 0).

Incubation Conditions of Solvent-Delivery Dosing System. The in vitro biotransformation rates obtained from the solvent-free sorbent-phase dosing experiments were compared with rates measured using a conventional solvent-delivery dosing system, in which test chemicals dissolved in solvent (acetonitrile) were added directly to the incubation medium. The same S9 preparations were used in both sorbent-phase dosing and solvent-delivery dosing experiments. Identical incubation conditions were used. Briefly, 0.5 mL incubation mixture in 2 mL vials was preincubated at 37 °C for 5 min, and 2.4 μL of test chemicals dissolved in acetonitrile was added to initiate the reactions. Final concentrations of chrysene, benzo[a]pyrene, and PCB 153 were 0.6, 1.0, and 1.0 μM, respectively. The final acetonitrile concentration was <0.5% (v/v), a concentration demonstrated to have no effect on five major cytochrome P450 activities in rat liver microsomes.[24] Incubations were carried out in triplicate experiments at 37 °C, and reactions were terminated at 5, 10, 15, 30, 45, 60, 75, 90, 105, and 120 min by adding 1 mL ice-cold hexane to the incubation medium to stop the reactions. The vials were stored on ice until chemical extraction.

Incubation Conditions for Protein Content Studies. To test the effect of altering protein content on the fraction of unbound substrate and the in vitro biotransformation rate, we conducted sorbent-phase dosing experiments as described above using 1.2–12 mg S9 protein per incubation by diluting S9 homogenates. Incubations were carried out in triplicate experiments at 37 °C, and reactions were terminated at 2.5, 5, 10, 15, 30, 45, 60, 75, 90, 105, and 120 min as described earlier.

Chemical Extraction. Prior to chemical extraction of the films, internal standards (0.21 nmol chrysene-d^{12} for benzo[a]pyrene and chrysene; 0.14 nmol PCB 155 for PCB 153) were added to the hexane extraction solvent. Test chemicals remaining in the film after the termination of the incubation were extracted into the hexane by vortexing the vials vigorously for 30 s. Extracts were transferred to a 2 mL autosampler vial for GC/MS analysis. The extraction efficiencies of the three test chemicals from EVA were 102.4 ± 1.4% (mean ± SD).

For extraction of test chemicals from the incubation medium, after the addition of internal standards (0.21 nmol chrysene-d^{12} for benzo[a]pyrene and chrysene; 0.14 nmol PCB 155 for PCB 153), vials containing S9 plus hexane were vortexed for 90 s. The vials were then centrifuged at 800 x g for 10 min (Thermo IEC Centra-CL2). The upper organic layer was transferred to a 2 mL autosampler vial for GC/MS analysis. The extraction efficiencies were 87.8 ± 1.5, 77.5 ± 4.4, and 63.7 ± 13.1% (mean ± SD) for chrysene, benzo[a]pyrene, and PCB 153, respectively.

Data Analysis. To determine the mass-transfer rate constants (k_1, k_2) and in vitro biotransformation rates (k_r) and their 95% confidence intervals from the results of sorbent-phase dosing experiments, a procedure for fitting the experimental data using a nonlinear regression[38] and a Runge–Kutta numerical differential equation solver[39] using MATLAB R2009a (Mathworks, Natick, MA) was developed as described in the Supporting Information.

To derive the biotransformation rate constants in solvent-delivery dosing experiments, the declining concentrations in the incubation medium over time were fitted by a first-order kinetic model:

$$\frac{dC_m}{dt} = -k_r \cdot C_m \quad (6)$$

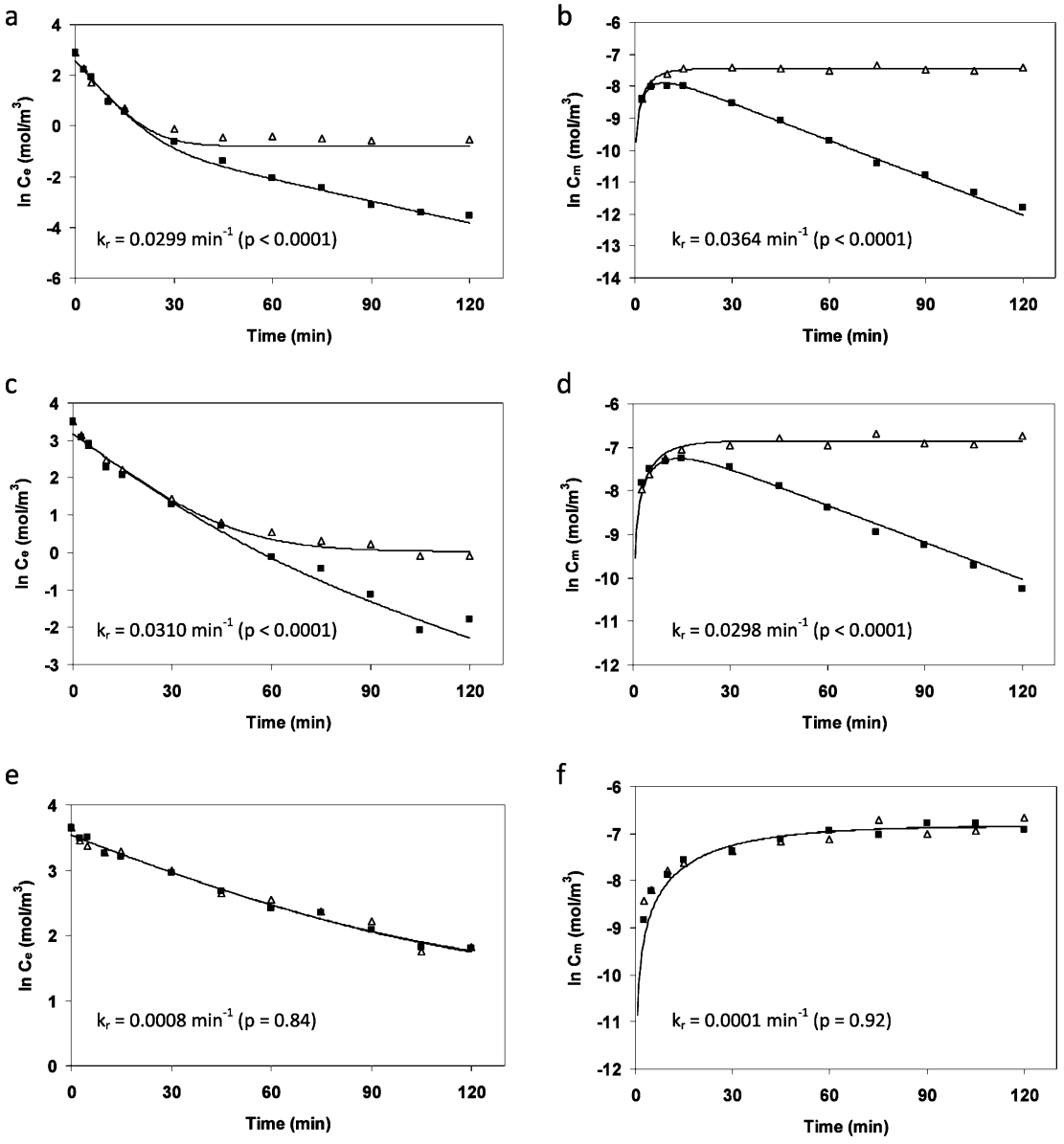

Figure 3. Concentration−time profiles in the EVA thin film sorbent phase (left) and in the incubation medium (right) containing active (solid squares) or inactive (open triangles) male Sprague−Dawley rat liver S9 homogenate (no cofactor control) using the sorbent-phase dosing approach for chrysene (a,b), benzo[a]pyrene (c,d), and PCB 153 (e,f). Solid lines represent nonlinear regressions. Data from one of three experiments are shown.

where C_m is the control-corrected chemical concentration in the incubation medium (μM); and k_r is the apparent first-order biotransformation rate constant (min^{-1}). k_r was estimated using a linear regression from the slope of ln ($C_m/C_{m,t=0}$) vs time (i.e., ln ($C_m/C_{m,t=0}$) = $-k_r \cdot t$), where $C_{m,t=0}$ is the initial dosing concentration of the chemical in the incubation medium (μM).

RESULTS AND DISCUSSION

Sorbent-Phase Dosing. Figure 3 illustrates that the concentrations of chrysene, benzo[a]pyrene, and PCB153 in thin films exposed to inactive no-cofactor control S9 liver homogenates declined over time to reach plateau concentrations for chrysene and benzo[a]pyrene, but not for PCB153, as the test chemicals transferred from the thin film into the liver homogenates. Corresponding concentrations in the inactive liver homogenates increased over time reaching apparent steady-state concentrations. Concentrations in the thin films exposed to active liver homogenates showed a continuous decline in concentration over time. Corresponding concentrations in the active liver homogenates increased over time, as the test chemical transferred from the thin film to the liver homogenate, then reached a maximum when the net mass transfer rate from film to medium equaled the biotransformation rate and finally declined (except for PCB153) when the biotransformation rate exceeded the net film to medium mass transfer rate. Concentrations of benzo[a]pyrene in the homogenate were well below the reported Michaelis−Menten constant K_M of 14.1 and 14.6 μM in rat liver S9.[40,41]

Chrysene and benzo[a]pyrene concentrations in the film and the liver homogenate showed highly significant differences between the test (active liver homogenate) and control (inactive liver homogenate) as demonstrated by the p-values (<0.0001) of the k_r nonlinear regression determinations (Figure 3). Statistically significant differences ($p < 0.05$) in concentrations between the

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control and test were not observed for PCB153 concentrations in the films or liver homogenates. Hence, PCB153 does not appear to be biotransformed at a significant rate, which is consistent with results from other studies.[42]

Figure 3 shows that the initial concentrations of benzo[a]-pyrene, chrysene and PCB 153 in test and no-cofactor control experiments were the same, indicating a similarity in the chemical exchange kinetics in the control and test systems. Such agreement was not observed when using controls prepared by heat treatment (Figure S1 in Supporting Information). This indicates that heat treatment affects the diffusion characteristics of the liver homogenate resulting in a change in the film-to-liver homogenate exchange kinetics between control and test which interferes with the measurement of k_r. For this reason, only data from the no-cofactor-controls were used for further analysis.

Figure 4 illustrates that for all test chemicals, k_1 was greater than k_r. This confirms that the delivery of the test chemicals from the sorbent phase to the liver homogenate was not the rate-limiting step, making it possible to determine k_r from concentration measurements in the thin films. Figure 4 also shows that the results from the three independent experiments were highly reproducible and that similar determinations of k_1, k_2, and k_r were obtained for each of the test chemicals by analyzing the chemical concentrations in the film only, the liver homogenate only, or the combined data set of concentrations in film and liver homogenate. This indicates that k_r can be determined by measuring either the concentrations in the film or the liver homogenate or both.

Figure S2 in the Supporting Information illustrates that the rate of chemical delivery from the thin film to the liver homogenate (k_1) decreased with increasing hydrophobicity (log K_{OW}) of the test chemical. A similar trend was observed for k_2. This means that with increasing hydrophobicity, it becomes more difficult to measure high biotransformation rates by analyzing the chemical concentrations in the EVA thin film. Since the measurement of biotransformation rate from concentrations in the liver homogenate medium is not directly affected by the rate of chemical delivery from film to medium, high biotransformation rates for very hydrophobic chemicals can still be measured by analyzing chemical concentrations in the incubation medium.

Solvent-Delivery Dosing. Figure 5 illustrates that in solvent-delivery dosing experiments, concentrations of chrysene and benzo[a]pyrene in rat liver S9 declined in a log−linear fashion over the course of the incubation period, while the concentrations of PCB153 did not. Linear regressions showed highly significant declines ($p < 0.05$ for the slopes) for chrysene and benzo[a]pyrene but not for PCB153. The apparent biotransformation rate constants (k_r) determined in triplicate experiments using no-cofactors and heat-treated controls are shown in Figure 4. Differences in the determination of k_r between using the two controls were not statistically significant ($p > 0.05$), indicating that in solvent based substrate delivery, the determination of in vitro biotransformation rates was not affected by the heat treatment of the homogenate.

Figure 4 shows that the in vitro biotransformation rates for both chrysene and benzo[a]pyrene using sorbent-phase dosing were two times greater ($p < 0.05$) than those derived from the solvent-delivery dosing experiments using the same liver preparation. The higher in vitro biotransformation rates obtained with the sorbent-phase dosing system may be attributable to (i) lack of enzyme inhibition by a spiking solvent, (ii) low initial

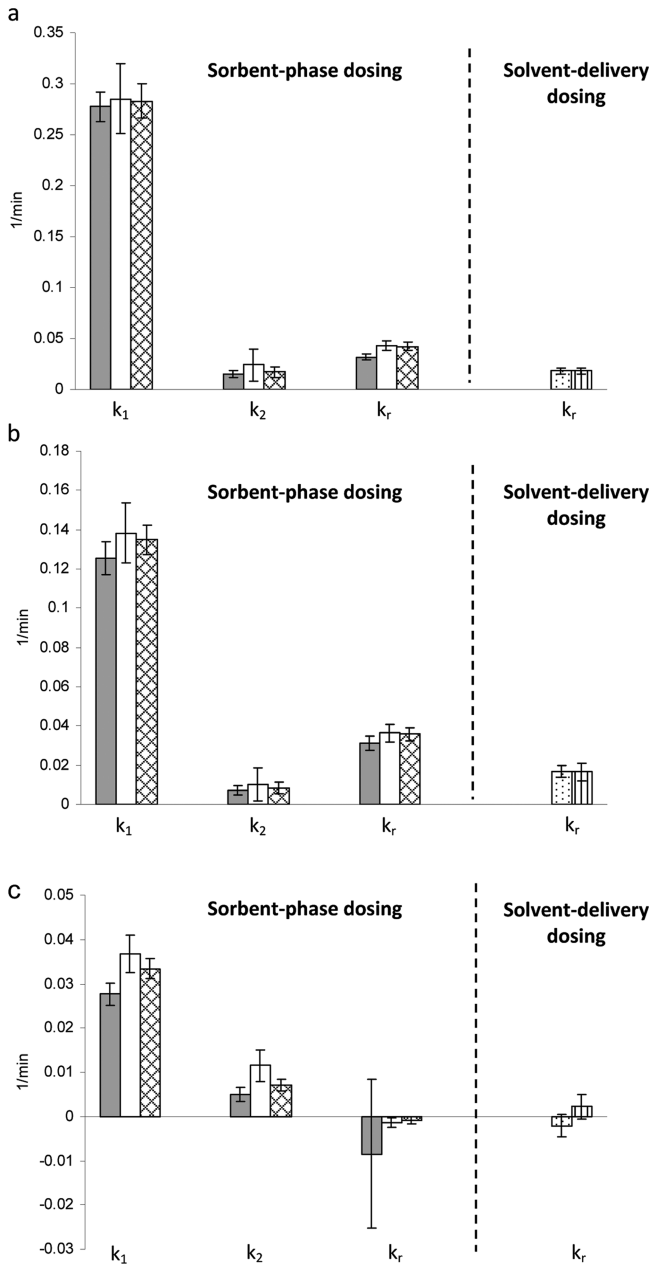

Figure 4. Measured mass-transfer rate constants (k_1 and k_2) and in vitro biotransformation rates (k_r) determined in sorbent-phase dosing experiments ($n = 3$) and solvent-delivery dosing experiments ($n = 3$) for chrysene (a), benzo[a]pyrene (b), and PCB 153 (c). Results from sorbent-phase dosing experiments were obtained from concentrations in thin films only (filled bars), the incubation medium only (empty bars), or the combined data set of thin film and incubation medium concentration data (cross-hatched bars) using the no-cofactors control. k_r values obtained from the solvent-delivery dosing experiments were analyzed using the no-cofactor control (dotted bars) or the heat-treated control (striped bars). Results were obtained from three independent experiments and error bars denote 95% confidence intervals.

substrate concentrations in the liver homogenate thereby reducing the potential for enzyme saturation and preventing precipitation (e.g., microcrystals) of the hydrophobic substrates[21] which can limit access of the substrate to enzymes, and (iii) reduction of enzyme inhibition by metabolic products because of their diffusion into the thin film. Studies have shown that metabolism of benzo[a]pyrene in rats can be inhibited by

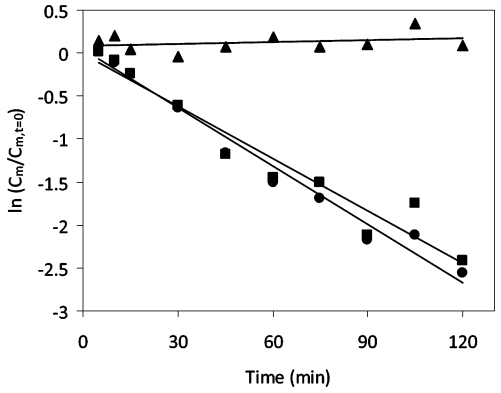

Figure 5. Concentration–time profiles expressed as the ratio of chemical concentrations in the incubation medium of the test and control for chrysene (●),benzo[a]pyrene (■), and PCB 153 (▲) in rat liver S9 homogenate using solvent-delivery dosing and no-cofactor controls. Results represent one of three independent experiments.

several of its metabolic products.[43−45] In addition, two-phase bioreactors enhance microbial biotransformation by in situ removal of inhibitory products by the organic phase.[46,47]

The measured apparent in vitro half-life (ln $2/k_r$) for benzo[a]pyrene was 22 min using sorbent-phase dosing compared to 41 min using solvent-delivery dosing. These values are consistent with published in vitro half-lives of benzo[a]pyrene in rat liver microsomes (30 min)[44,45] and rat hepatocytes (60 min).[7] However, biotransformation rates expressed as clearance rates in units of mL h^{-1} mg $protein^{-1}$ encompass a wide range of values from 0.63 to 19.4 mL h^{-1} mg S9 $protein^{-1}$ (Table S1 in Supporting Information). Using the sorbent-phase dosing system, we obtained 0.2 mL h^{-1} mg S9 $protein^{-1}$. The differences in measured biotransformation rates likely arise due to interlaboratory differences in experimental approach such as monitoring the rate of metabolite formation versus substrate depletion, and differences in protein concentrations and consequently, in the fraction of unbound chemical substrate used in the in vitro system. The relationship between in vitro biotransformation rate and concentrations of protein and unbound chemical fractions is discussed in the next section.

Unbound Chemical Substrate Fraction. The unbound fractions of chrysene, benzo[a]pyrene and PCB153 in the incubation medium were determined from the concentrations in the film and the incubation medium in the control experiment at steady-state as $C_e/(K_{ew}\cdot C_m)$ where K_{ew} was $10^{6.53}$ for chrysene, $10^{6.77}$ for benzo[a]pyrene and $10^{8.10}$ for PCB153.[35] Figure 6 illustrates that increasing the amount of liver homogenate protein 10-fold per incubation (from 1.2 to 12 mg) resulted in a statistically significant decline in the fraction of unbound chrysene and benzo[a]pyrene in the liver homogenate from approximately 0.2% to 0.025% (for chrysene) and from 0.12% to 0.018% (for benzo[a]pyrene). However, Figure 6 also shows that while the fraction of unbound chemical decreased, there was no statistically significant change in the value of k_r. The significance of the slope of the linear regression of k_r versus protein content was characterized by probability values of p = 0.93 for chrysene and p = 0.09 for benzo[a]pyrene. It appears that for the very hydrophobic chemicals in this study, a 10-fold increase in enzyme activity in incubations (which elevates biotransformation rates) was associated with a similar reduction in the unbound substrate fraction (which reduces biotransformation rates), causing no statistically significant changes in the apparent in vitro biotransformation rates with increasing protein content. A similar observation was observed in a previous study with very hydrophobic substrates.[48] These observations are consistent with the theory expressed by eq 3. These findings imply that protein normalization of measured biotransformation rate constants of very hydrophobic substances can produce substantial error in the measurement of k_r and that the large variation in observed in vitro intrinsic clearance rates of benzo[a]pyrene among different studies documented in Table S1 of the Supporting Information may be partly explained by protein normalization of measured biotransformation rates. These findings emphasize the importance of specifying both protein content and unbound substrate fraction in the measurement and reporting of in vitro biotransformation rates for very hydrophobic substances. The study indicates that the intrinsic in vitro biotransformation clearance rate of the unbound chemical is remarkably fast at rates of 10.9 ± 1.5 mL·min^{-1}·mg S9 $protein^{-1}$ (mean ± SD) for chrysene and 15.3 ± 4.1 mL·min^{-1}·mg S9 $protein^{-1}$ (mean ± SD) for benzo[a]pyrene and that the binding of the very hydrophobic chemicals to liver homogenate constituents other than the active sites of biotransforming enzymes exerts a large influence on the apparent biotransformation rate.

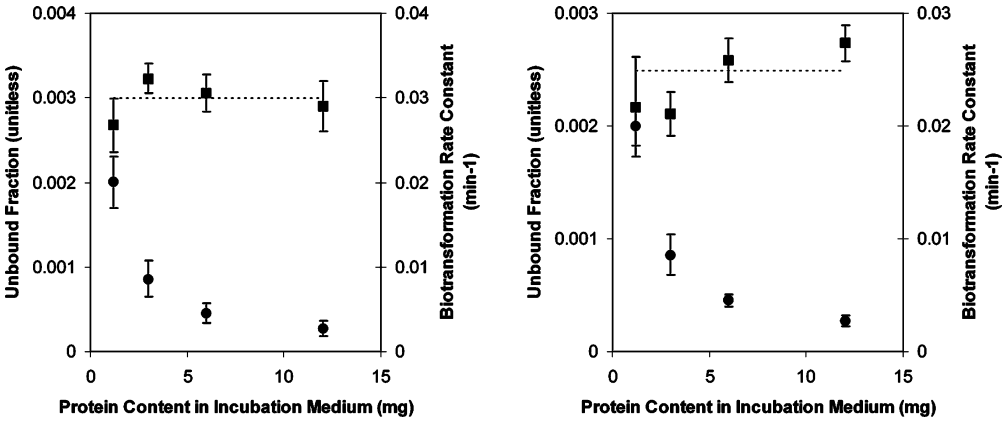

Figure 6. Relationship between the rat liver S9 protein content (mg) in the incubation medium and the unbound fraction (unitless) in the incubation medium (●, left axis) and the apparent biotransformation rate constant k_r (min^{-1}) (■, right axis) for chrysene (left) and benzo[a]pyrene (right). Error bars denote 95% confidence intervals. Dotted lines represent the average apparent biotransformation rate constant.

Method Application. The implementation of effective bioaccumulation screening under the UN Convention on Persistent Organic Chemicals, REACH in the European Union, CEPA In Canada and TSCA in the U.S. requires access to methods that can, with relative ease and low cost, determine biotransformation rates of many commercial chemicals.[3] Of particular importance are methods for chemicals with very high octanol−water and octanol-air partition coefficients as these chemicals have an intrinsic potential for food-web biomagnification. In addition, methods tailored for mammalian species are important because mammals occupy high trophic positions in food-webs and for many chemicals, bioaccumulation measurements in fish do not provide accurate estimates of bioaccumulation in mammals.[4,49] The extreme hydrophobicity and very low aqueous solubility of chemicals with a high bioaccumulation potential can pose methodological challenges especially if the method involves chemical dissolution in an aqueous medium. The sorbent-phase dosing technique explored in this study may reduce some of these challenges by eliminating the need for the addition of chemical carrier solvents and by helping to maintain first order kinetics of biotransformation by the low initial substrate concentrations in the incubation medium. The experimental analysis of chemical concentrations in the thin film does in many cases not require the type of analytical clean up procedures often associated with the extraction and analysis of organic chemicals in complex biological matrices like liver homogenates. Measurement of chemical concentrations in the thin films also provides a relatively simple method for measuring the unbound chemical fraction in the incubation medium, which is important for in vitro to in vivo extrapolations. The findings of this study suggest that the thin-film sorbent-phase dosing approach may be a simple and fast screening tool for measuring the in vitro biotransformation rates of commercial substances with a high bioaccumulation potential in mammalian species.

One of the key limitations of sorbent-phase dosing technique is the use of an appropriate control. The no-cofactor-control may be appropriate for chemicals whose biotransformation is primarily mediated by cytochrome P450 enzymes, such as the oxidations of many polyaromatic hydrocarbons (PAHs). However, the heat or chemically treated controls may be necessary for biotransformation reactions mediated by enzymes that do not require NADPH or other cofactors (e.g., soluble enzymes in the liver cytosol). This study showed that heat treatment of the incubation medium affects the film-to-medium exchange kinetics, causing differences in the concentration time course between test and control unrelated to biotransformation. Future studies are needed to develop strategies for using heat-treated controls or for finding alternative chemical controls (e.g., mercury or sodium azide treatment) for assessing the biotransformation ability of chemicals that are not biotransformed by cytochrome P450.

■ ASSOCIATED CONTENT

Supporting Information

(i) Details of the chemical substances used, the preparation of rat liver S9 homogenates and GC/MS analysis; (ii) a statistical method for the analysis of concentration time curves; (iii) concentration−time profiles in the EVA thin film and incubation media containing active and heat treated rat liver S9; (iv) data on the relationship between log K_{OW} and mass-transfer rate constants k_1 and k_2 in the thin-film sorbent-phase dosing system; and (v) a compilation of in vitro intrinsic clearance rates of benzo[a]pyrene in rat liver S9 homogenates and microsomes and (vi) supporting references. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: gobas@sfu.ca.

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