Assessment of Metabolic Stability Using the Rainbow Trout (*Oncorhynchus mykiss*) Liver S9 Fraction

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ABSTRACT

Standard protocols are given for assessing metabolic stability in rainbow trout using the liver S9 fraction. These protocols describe the isolation of S9 fractions from trout livers, evaluation of metabolic stability using a substrate depletion approach, and expression of the result as in vivo intrinsic clearance. Additional guidance is provided on the care and handling of test animals, design and interpretation of preliminary studies, and development of analytical methods. Although initially developed to predict metabolism impacts on chemical accumulation by fish, these procedures can be used to support a broad range of scientific and risk assessment activities including evaluation of emerging chemical contaminants and improved interpretation of toxicity testing results. These protocols have been designed for rainbow trout and can be adapted to other species as long as species-specific considerations are modified accordingly (e.g., fish maintenance and incubation mixture temperature). Rainbow trout is a cold-water species. Protocols for other species (e.g., carp, a warm-water species) can be developed based on these procedures as long as the specific considerations are taken into account.*Curr. Protoc. Toxicol.* 53:14.10.1-14.10.28. © 2012 by John Wiley & Sons, Inc.

Keywords: liver S9 fraction • rainbow trout • metabolism • in vitro assay • fish • metabolism • CYP450 • phase I and II metabolism enzymes

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INTRODUCTION

Hepatic metabolism of xenobiotic compounds by fish has been studied using several in vitro test systems including precision-cut liver slices, isolated hepatocytes, subcellular fractions (microsomes and S9), and recombinant enzyme systems (Schlenk et al., 2008). Each system has advantages and disadvantages that dictate its utility for specific applications. For example, recombinant systems can be used to evaluate questions (e.g., substrate specificity, kinetic parameters) pertaining to a specific cytochrome (CYP) P450 enzyme. However, this system requires substantial technical expertise to develop and use. Moreover, the resulting information may provide little insight regarding other enzymes responsible for metabolism of a particular substrate. Liver slices and isolated hepatocytes contain the full complement of metabolizing enzymes as well as membrane-bound drug transporters but are technically demanding to prepare and use. Microsomal fractions provide an intermediate system with respect to ease of preparation, and are well suited to the study of compounds principally metabolized by CYP enzymes.

The liver S9 fraction is relatively easy to prepare and use, and contains both cytosolic and microsomal enzymes. This fraction contains phase I (e.g., CYP) and phase II [e.g., sulfotransferase (SULT), uridinediphosphate-glucunosyltransferase (UGT), glutathione S-transferase (GST)] enzymes involved in metabolism of drugs and xenobiotics. These features make the S9 system a good choice for studies designed to assess hepatic clearance by fish, particularly if the enzymes responsible for metabolism of a particular compound are unknown. Although the S9 fraction was originally named after the centrifuge speed ($9000 \times g$) used in its preparation, recent investigators have employed higher centrifuge speeds in an effort to obtain a "cleaner" preparation; hence, the appearance of terms such as S10 and S13. In this unit, the term S9 is applied broadly to subcellular preparations obtained using a single, low-speed (commonly 12,000 to 13,000 $\times$ g) centrifugation step.

Numerous methods for preparing fish liver S9 fractions have appeared in the literature, and the protocol given here shares many features with these published procedures. In this application, however, the method to support a specific purpose, that of rapidly estimating metabolic stability of a chemical in rainbow trout S9 fractions by an in vitro method, which can be performed in a single day, as opposed to upwards of 60 days for in vivo approaches, has been adapted. Basic Protocol 1 describes isolation of the S9 fraction from trout liver, assessment of metabolic stability using a substrate depletion approach, and expression of the result as in vivo intrinsic clearance. Potential applications of these methods in the field of ecotoxicology include prediction of metabolic impacts on chemical accumulation by fish (Nichols et al., 2007, 2009), evaluation of emerging chemical contaminants (e.g., pharmaceuticals in municipal waste water), and improved interpretation of in vivo toxicity testing results.

CAUTION: All tissues should be handled with caution due to possible presence of infectious agents. Wear appropriate laboratory coat, gloves, and eye protection during all laboratory operations. Read the Material Safety Data Sheet (MSDS) for each organic solvent and test compound, and employ recommended safety measures. Bulk solvents should be handled in a fume hood. Liquid nitrogen and dry ice should be handled using cryogenic gloves in a well-ventilated area.

NOTE: This protocol uses live animals and should be reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) or equivalent of the company or research center where the procedure will take place.

RAINBOW TROUT LIVER S9 FRACTION PREPARATION

The flow diagram shown in Figure 14.10.1 describes the general steps for preparing trout liver S9 fractions, conducting metabolic stability assays, and calculating in vivo intrinsic clearance. In fish, as in mammals, the amounts and activities of liver biotransformation enzymes varies among individuals. To obtain a representative measure of activity, it is necessary to pool S9 fractions from several (typically four to five) individuals. Detailed information pertaining to the selection and use of fish is provided in the Commentary.

Throughout this protocol we use 12°C as a standard temperature for holding trout and conducting metabolic stability studies. Of greater importance, however, is the need to conduct metabolism studies at the temperature to which fish have been acclimated. This is because the activities of metabolic enzymes in fish tend to exhibit "ideal" temperature compensation (Karr et al., 1985; Carpenter et al., 1990), i.e., metabolic activity changes with acclimation temperature resulting in activities that are similar among animals, regardless of their holding temperature. At each point in this protocol, the acclimation temperature may be substituted for the stated value of 12°C, if different.

It is strongly recommended to use fresh rather than frozen tissue. In principle, frozen livers can be thawed at a later date and used to prepare S9 fractions. This approach has the advantage that livers collected at one location (e.g., a fish hatchery) can be transported to another location for processing (e.g., a laboratory), thereby eliminating the need for fish holding facilities at the site of S9 preparation. Previous work has shown, however, that freezing and thawing fish liver tissue reduces the activities of CYP enzymes associated with the microsomal fraction (Förlin and Andersson, 1985; Lindström-Seppä and Hänninen, 1988). While these earlier studies were conducted using livers that were excised and immediately frozen (whole or after being cut into pieces), similar results have been obtained in the authors' laboratories even when livers are cleared of blood before freezing (K. Johanning, A. Adekola, C. Eickhoff, and J. Nichols, unpub. observ.).

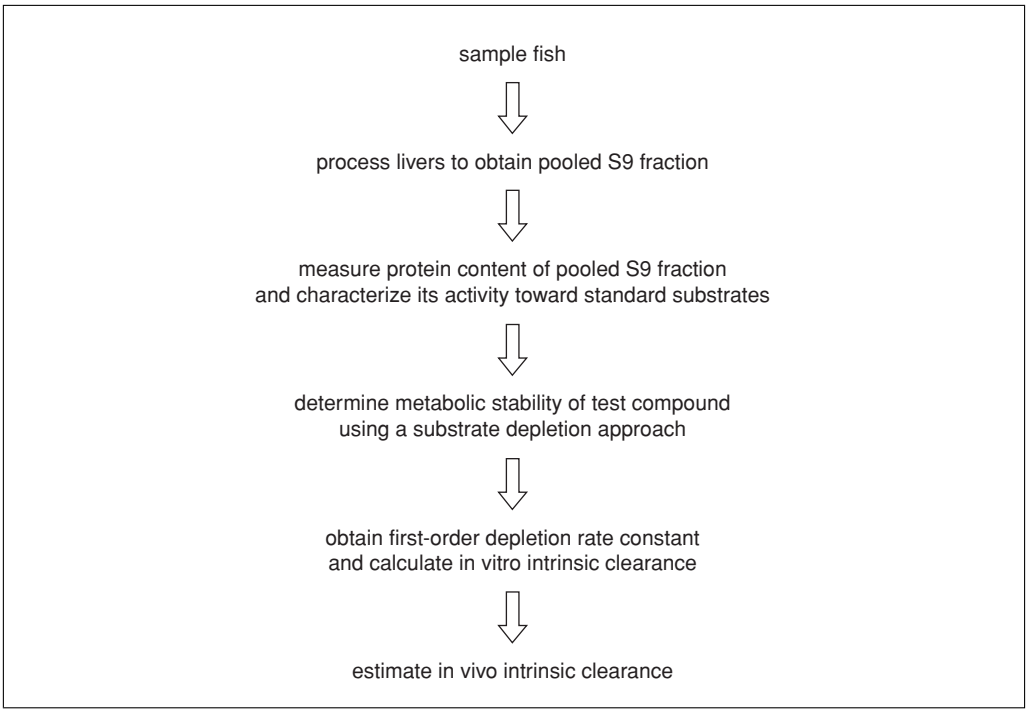

Figure 14.10.1 Graphic representation of steps in the protocol, from fish sampling to calculation of in vivo intrinsic clearance.

Materials

Rainbow trout (1 to 1.5 years old; 400 to 600 g body weight)
Tricaine methanesulphonate (MS-222; see recipe)
$NaHCO_3$
Clearing buffer (solution A, see recipe)
Homogenization buffer (solution B, see recipe), ice cold
Liquid nitrogen

Fish net
10-liter plastic bucket
Fish knife
Paper towel or absorbent paper
Digital balances for 1- to 100-g and 100- to 3000-g quantities
Surgical scissors and forceps
Silk suture material (4/0; Roboz, cat. no. SUT-15-2)
Safety-winged infusion needle set, 23-G × $^3/_4$-in. (VWR, cat. no. 14229-297)
30-ml disposable plastic syringes
6-cm glass petri dishes, pre-chilled
Analytical balance (for milligram quantities)
50-, 150- and 250-ml glass beakers
30-ml Wheaton Potter-Elvehjem mortar with Teflon pestle (VWR, cat. no. 62400-788), ice cold
Multi-speed bench-top drill press (e.g., Ryobi DP102L)
50-ml round-bottom centrifuge tubes (e.g., Nalgene 50-ml round-bottom polypropylene copolymer centrifuge tubes; VWR, cat. no. 21010-829)
Two-pan balance
Refrigerated centrifuge (e.g., Beckman J2-21 or J2-MC centrifuge equipped with a fixed-angle JA-17 or JA-10 rotor)
Pasteur pipets
1.8-ml working volume cryogenic storage tubes (e.g., Thermo Scientific Nunc; Cole-Palmer, cat. no. EW-03755-10)
Plastic freezer bags

Obtain fish sample and clear and excise liver

1. Obtain juvenile rainbow trout, 1.0- to 1.5-years old, weighing 400 to 600 g.

   Rainbow trout are typically raised at a water temperature of 10° to 15°C. The holding water temperature in the laboratory should be close to that of the rearing water and maintained at ±2°C. Additional information regarding fish selection and treatment are provided in the Commentary.

2. Acclimatize fish in the laboratory for at least 3 weeks prior to use. Measure water chemistry characteristics and record at periodic intervals, including pH, hardness (total alkalinity as mg/liter $CaCO_3$), dissolved oxygen (mg/liter, converted to percent saturation), and total ammonia (mg/liter).

3. Prepare a record (see Fig. 14.10.2 for an example) prior to fish sampling and record fish information, maintenance conditions, and individual fish observations.

4. Fast fish for at least 24 hr prior to sacrifice.

5. Net a fish and transfer to a 10-liter bucket containing 6 to 8 liters of anesthetic solution (MS-222, plus a previously determined amount of $NaHCO_3$).

   The addition of MS-222 to water reduces its pH. Adding $NaHCO_3$ will maintain the pH close to that of the holding water. See Reagents and Solutions for more information. The same MS-222 solution may be used to anesthetize up to three to five fish without loss of anesthetic efficacy.

Species (e.g., *Oncorhynchus mykiss*): _____

Strain (e.g., Kamloops): _____

Fish source (e.g., hatchery name): _____

Photoperiod regime (e.g., natural photoperiod): _____

Water temperature (°C): _____

Water source (e.g., well): _____

Water flow rate (liter/min): _____

Fish holding density (kg/liter tank volume): _____ or

Number fish/tank: _____

Feeding regime (e.g., % body wt./day): _____

Feed type (e.g., Nelson's Silver Cup trout feed): _____

Fish no.	Fish wt. (g)	Sex (female or male)	Liver wt. and HSI (g liver and g liver/ g fish wt.)	Gonad wt. and GSI (g gonad and g gonad/ g fish wt.)
1				
2				
3				
4				
5 and so on				

Figure 14.10.2 Record of fish information, maintenance conditions, and individual fish observations.

6. Once the opercular movements of the fish have slowed (~1 min after immersion in the MS-222 solution), euthanize the animal with a sharp blow to the head. Proceed quickly with the following steps.

   Alternatively, euthanasia may be accomplished by severing the spinal cord and pithing the animal with a metal wire. It is important, however, to perform this step rapidly so that blood flow within the animal is maintained, thereby facilitating cannulation of the portal vein and removal of blood from the liver tissue (steps 10 and 11). When performing this step for the first sampled animal, record the time. This can be used as the "starting" time to determine the total length of time required to prepare and freeze S9 fractions. The "ending" time is the time that the last aliquot of S9 protein is snap frozen.

7. Place paper towels on top of a digital balance. Tare the balance, weigh the fish to the nearest gram, and record.

8. Transfer fish to a flat working surface covered with paper towels or other absorbent material. Make a longitudinal incision with a pair of scissors, extending from the cloaca forward to the isthmus (lower jaw region). Make two additional transverse incisions extending dorsally just behind pectoral fins, exposing the internal organs (Fig. 14.10.3).

Figure 14.10.3 Incisions required exposing the internal organs of the fish after it has been euthanized. The fish in this picture is sexually mature and is shown for illustrative purposes only. Sexually immature animals should be used to obtain liver S9 fractions.

9. Sex the animal and record this information. Depending on the number of fish of each sex that have been sampled to this point, process the animal or discard.

   The animal's sex is determined by examining its gonads. In sexually immature fish, the testes appear as two threads of pinkish tissue, located just below the ventral surface of the kidney and running most of the length of the peritoneal cavity. The ovaries will be similar in size to the testes or somewhat larger. An examination of the ovaries under a dissecting microscope will reveal a granular appearance. Descriptions of the testes and ovaries in trout at different stages of sexual maturity are provided by Billard (1992).

   Because the fish being used are not sexually mature, it is not possible to determine their gender before processing. In practice, animals are sampled until the desired number of each sex has been obtained (e.g., five). Random chance dictates that more than ten animals may be needed to satisfy this goal, possibly resulting in excess animals. If possible, these excess animals should be saved for another use, identified ahead of time. If it is not possible to obtain the desired number of animals of each sex in an acceptable period of time (~1 hr), it may be necessary to process the livers already collected. This is a judgment call that must be made by the researcher. Thus, a pooled homogenate obtained from four fish in 1 hr may be preferred to a pooled homogenate from five fish collected over 2 hr. It is common for the sex ratio of a fish population to be skewed somewhat. However, a sex ratio that is strongly skewed in either direction should be viewed with suspicion and may necessitate obtaining a new group of animals.

10. Loosely loop silk suture material (4/0) under the blood vessel to be cannulated before inserting the needle. Cannulate the hepatic portal vein (Fig. 14.10.4) using a safety winged infusion needle set, 23-G × $^{3}/_{4}$-in., attached to a 30-ml syringe filled with ice-cold clearing buffer (solution A). Then draw the 4/0 silk suture tightly around the needle and tie off to prevent leakage of solution A from the insertion site.

    With practice, it is possible to perfuse the liver without using suture material to tie off the portal vein. Instead, the insertion point may be sealed by gently pinching the portal vein between the thumb and index finger. This technique saves time and may be easier for a single individual to perform.

11. Sever the hepatic vein(s) leading from the liver to the heart to permit drainage of the tissue.

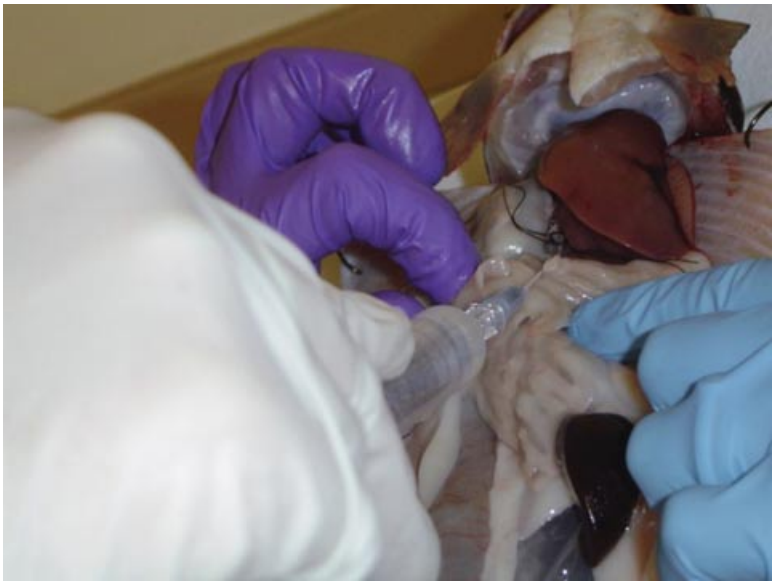

Figure 14.10.4 Cannulation of the hepatic portal vein to clear the liver of blood.

12. Perfuse the liver slowly (~10 to 15 ml/min) with 20 to 30 ml of solution A until the tissue is pale in color (i.e., blood is removed; Fig. 14.10.5). Keep solution A, associated syringes, and solution B on ice at all times before use.

    Gently massage the liver while perfusing to aid flow of the blood, especially areas where the blood is concentrated.

    Removal of blood is required to ensure that S9 fractions are free of blood-borne metabolizing enzymes (e.g., plasma proteases). Because S9 proteins degrade rapidly at room temperature, every effort should be made to carry out these procedures using pre-chilled solutions, instruments, and glassware.

13. Excise the liver and place on a chilled petri dish, taking care not to cut open the gall bladder (a thin-walled sac, usually containing dark green or brown bile).

14. Carefully remove the gall bladder by using scissors to sever the connective tissue that attaches it to the liver. Rinse the tissue with 5 to 10 ml of ice-cold solution A, kept on hand in a 30-ml syringe for this purpose.

    It is important that gall bladder bile does not contaminate the liver S9 fraction since this will denature and inactivate metabolic enzymes. This rinse step is employed as a precaution to deal with possible contact of the liver with small amounts of bile. Livers that come in contact with large amounts of bile should be rejected from further processing if possible. Otherwise rinse with solution B thoroughly and quickly to wash off the bile contents if the exposure is minimal.

15. Blot the liver on a paper towel to remove excess fluid and weigh to the nearest 0.01 g. Determine the hepatosomatic index (HSI) of the donor animal, calculated as the liver weight divided by the whole animal weight. Record both the liver weight and HSI (see Fig. 14.10.2).

16. Place the liver in a 250-ml beaker containing 150 ml ice-cold homogenization buffer (solution B).

17. Remove the gonads (ovaries or testes) in their entirety and weigh to the nearest 0.01 g. Determine the gonadosomatic index (GSI) of the donor animal, calculated as the gonad weight divided by the whole animal weight. Record both the gonad weight and GSI (see Fig. 14.10.2).

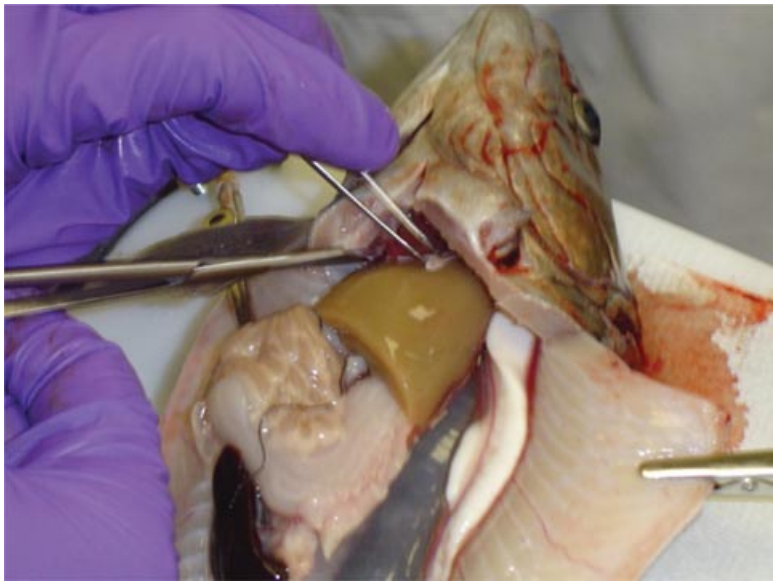

Figure 14.10.5 Blanched appearance of a liver that has been successfully cleared of blood.

18. Repeat steps 5 to 17, separating livers into two beakers based on the sex of donor animals, until the desired number of livers is obtained.

    It is important to proceed quickly. The total length of time from netting a fish to placing a liver in solution B should be <15 min. Researchers should practice these procedures before collecting S9 fractions for experimental purposes.

Process and prepare liver S9 fractions

19. Remove a liver from solution B, blot with a paper towel, and weigh to the nearest 0.01 g. Transfer the liver to a 50-ml beaker that has been kept on ice and add an equivalent weight of ice-cold solution B. Cut the liver into small (<0.5-cm^2) pieces with a pair of scissors.

20. Transfer minced tissue in solution B to a Potter-Elvehjem mortar (pre-chilled and kept on ice). Rinse the beaker with an additional volume (equivalent to the liver weight) of solution B and transfer this volume along with any remaining tissue to the mortar. Homogenize the tissue using a Teflon pestle (normal clearance 0.1 to 0.15 mm) attached to a bench-top drill press.

    This should be done by forcing the pestle slowly (10 to 15 sec per stroke) to the bottom of the mortar. Repeat this maneuver four to five times. Additional homogenization is not required and may cause protein denaturation. Avoid pushing the pestle into the glass mortar too hard as this may break it. The drill press should be set at low speed resulting in ~500 rpm. Wear leather gloves to protect hands.

21. Pour liver homogenate into a 50-ml round-bottom centrifuge tube (pre-chilled and kept on ice) designated for each sex. Repeat steps 19 and 20 until all livers have been processed. Balance each centrifuge tube against a second tube containing water or S9 homogenate using a two-pan balance or equivalent.

    Assuming the fish size range (400 to 600 g) and sample number (four to five) recommended here, the expected volume of raw liver homogenate is 50 to 70 ml. This will require the use of two centrifuge tubes per sex.

22. Centrifuge liver homogenates 20 min at 13,000 × *g*, 4°C.

23. Gently remove centrifuge tubes from the centrifuge. Depending on the lipid content of livers being sampled, a layer of yellow lipid may form on the surface of the supernatant; aspirate this layer with a Pasteur pipet and discard. Decant the remaining supernatant into a chilled 150-ml beaker taking care not to transfer any material from the pellet (this is the pooled S9 fraction).

    The pellet at the bottom of the centrifuge tube should be relatively firm and brown in color. A lighter-colored layer may form on the surface of the pellet.

24. Mix the pooled S9 fraction using a glass rod or equivalent, then transfer 0.5-ml aliquots of the solution to pre-labeled 1.8-ml cryogenic storage tubes and immediately snap freeze in liquid N_2 (or equivalent). Repeat until the entire S9 fraction for each sex has been distributed, stirring the solution between samples.

    It is extremely important to keep liver S9 fractions on ice at all times. Four to five fish of the recommended size will yield 35 to 45 ml of S9 fraction. Assuming that this is divided into 0.5-ml aliquots for storage, it will be necessary to pre-label 70 to 90 cryogenic storage tubes for each sex. Labeled tubes should be chilled before samples are added and stored not more than 24 hr in a −20°C freezer until just prior to use.

25. Collect samples in pre-labeled plastic freezer bags and transfer to liquid nitrogen or a −80°C freezer for storage (for at least 2 years).

26. Record the total number of tubes for each sex and multiply by the volume of S9 fraction distributed to each tube to calculate the total volume of S9 fraction obtained from a single sampling effort.

    This value is used in Basic Protocol 2, step 30, to calculate the concentration of S9 protein per gram of liver tissue.

IN VITRO DETERMINATION OF METABOLIC STABILITY AND EXTRAPOLATION TO IN VIVO INTRINSIC CLEARANCE

BASIC PROTOCOL 2

The flow diagram shown in Figure 14.10.6 describes the general steps for performing metabolic stability assays and calculating in vivo intrinsic clearance. Metabolic stability experiments are conducted using a substrate depletion approach wherein the metabolic rate is determined by measuring disappearance of parent chemical from the reaction vessel. The incubation system consists of pooled S9 protein suspended in 100 mM potassium phosphate buffer (solution C). Cofactors are added to support both Phase I and II metabolic pathways. The reaction is run at 12.0 ± 1.0°C and is initiated by the addition of test compound. A minimum of six sampling time points is recommended to develop a high-quality regression of log-transformed chemical concentration data (see step 28 below).

Preliminary incubations should be performed to establish reaction conditions and sampling time points appropriate to a given compound. Important variables include the starting concentration of test compound, the concentration of S9 protein in solution, and the total reaction run time. The protein content of a pooled S9 sample must be determined in advance to calculate the amount added to the reaction mixture. Additional preliminary experiments are conducted to characterize the activity of each pooled S9 sample using standard substrates (i.e., positive controls) for one or more Phase I and II metabolic reactions. Details pertaining to the determination of appropriate reaction conditions and use of positive controls are given in the Commentary.

The total incubation time for a substrate depletion experiment should not exceed 2 hr due to progressive loss of enzyme activity (typically 10% to 15% per hour, unpub. observ.). Because rates of biotransformation are temperature sensitive, the temperature at which the assay is carried out should be tightly controlled using a water bath or equivalent. Incubations conducted using heat-denatured S9 fractions are used to distinguish between

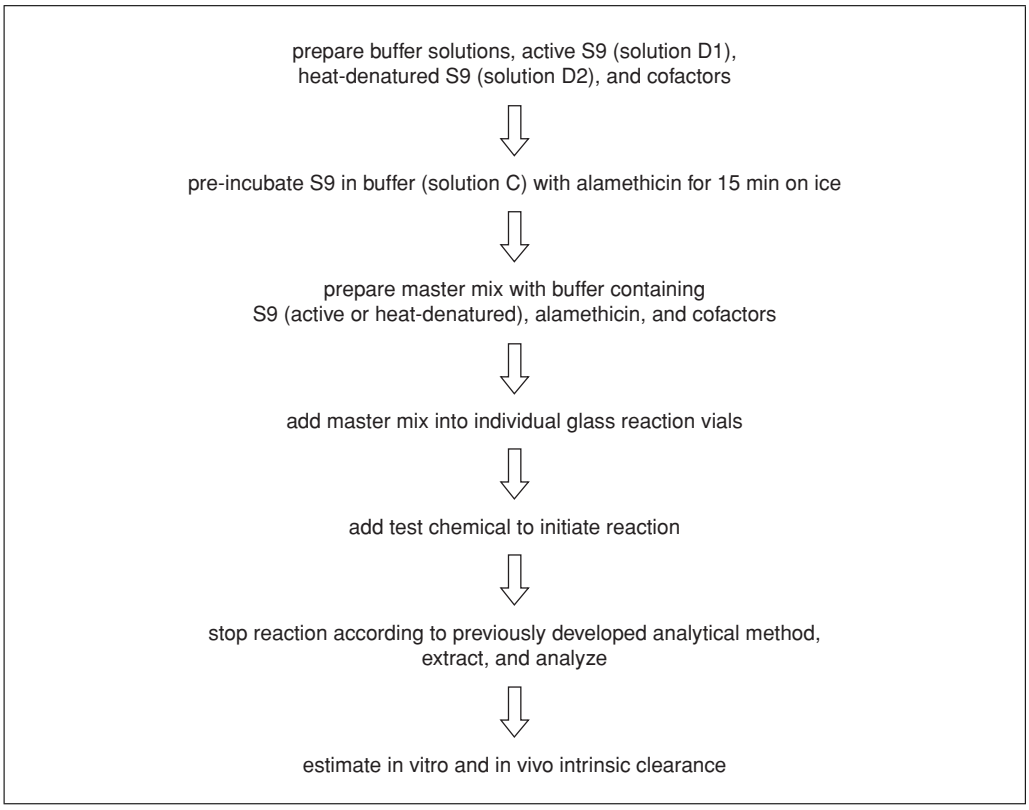

Figure 14.10.6 Graphic representation of the steps to determine metabolic stability of a test compound.

enzymatic metabolism and other potential loss processes including abiotic degradation, volatilization, and adsorption to the reaction vessel. Additional information pertaining to the selection and use of negative controls is provided in the Commentary. Analytical methods for the test substance must be developed and validated before conducting any substrate depletion studies.

Trout liver S9 incubations can be performed by adding one reagent at a time; however, a "pool approach" is recommended. With the pool approach all reagents are combined in an Erlenmeyer flask to create a "master mix." This solution is then distributed to each test tube before adding the test chemical in spiking solvent. Two master mixes are created by sequential addition of solutions E, F, G, H, and I to a protein solution, D1 (active) or D2 (heat-inactivated or denatured). Protein solutions are prepared by spiking active (solution D1) or denatured (solution D2) S9 protein into solution C. The volume of S9 fraction spiked into solution C depends on the measured protein content, which is determined in advance. Solution E contains alamethicin, which creates pores in the endoplasmic reticulum making UGT enzymes available to catalyze glucuronidation of appropriate substrates. Solutions F through I contain cofactors required to support Phase I (CYP-mediated) and II (sulfation, glucuronidation, and glutathione conjugation) metabolic reactions. The final concentrations for each of the co-factors in the incubation mixture are: 2 mM NADPH, 2 mM UDPGA, and 0.1 mM PAPS. The master mixes are created using chilled (4°C) solutions and kept on ice until used. A single-channel or repeater pipettor is used to dispense the master mixes into the reaction vessels. The samples are then allowed to equilibrate for 10 min at 12°C before initiating the reaction.

NOTE: The specific brands of equipment and reagents are only a recommendation; equivalent equipment or reagents may be substituted. It is the responsibility of the researcher to determine the substitute suitable for a particular application.

Materials

Rainbow trout liver S9 fraction (active and heat-denatured S9 fractions, see Support Protocol; prepared and frozen as described in Basic Protocol 1)
Commercial protein assay (e.g., Pierce BCA Protein Assay; Thermo Scientific, cat. no. 23227)
100 mM potassium phosphate buffer (solution C; see recipe)
Nicotinamide adenine dinucleotide 2′-phosphate, tetrasodium salt (NADPH; solution F; see recipe)
Uridine 5′-diphosphoglucuronic acid, trisodium salt (UDPGA; solution G; see recipe)
L-Glutathione (GSH; solution H; see recipe)
3′-Phosphoadenosine 5′-phosphosulfate (PAPS; solution I; see recipe)
Test compound
Spiking and extraction solvents appropriate for test compound of interest (HPLC-grade or better; see recipe)
Solution E: alamethicin in 2.5% methanol/97.5% solution C (see recipe)
96-well flat-bottom plates (e.g., Thermo Scientific, Nunc; VWR, cat. no. 269620)
Microplate reader with UV-visible spectrophotometer (e.g., Molecular Devices THERMOmax microplate reader)
pH meter (e.g., Accumet AB15+ and BioBasic pH/mV/°C meter, Fisher Scientific, cat. no. 13-636-AB15P)
Glass inserts for 96 deep-well format (e.g., Hirschmann glass inserts, VWR, cat. no. 89022-288) or alternative gas chromatography amber glass test tubes target DP T/S septa vials (National Scientific, cat. no. C400-2W)
Parafilm
Shaking water bath (e.g., Lab Companion 17-liter reciprocal shaking water bath; Cole Palmer, cat. no. EW-12054-00)
Circulating chiller for water bath (e.g., 6-liter refrigerated circulating bath; Cole Palmer, cat. no. EW-12108-00)
Holder for 96 glass inserts (e.g., VWR, cat. no. 89022-294)
25-ml Erlenmeyer flask
Vortex mixer (e.g., Thermo Scientific MaxiMix/vortex mixer, cat. no. 12-815-50)
250-ml beaker
Eppendorf Repeater Plus pipettor (e.g., Eppendorf, cat. no. 022260201)
Combitips for Repeater Plus pipettor (e.g., 0.2-ml volume, Eppendorf cat. no. 022266004)
Thermo Scientific Nunc 96-well cap mat (e.g., Fisher Scientific, cat. no. 12-565-559)
MultiTube vortex (e.g., Fisher Scientific, cat. no. 02-215-452), optional

Determine protein content

1. Thaw three S9 samples and determine their protein content using a commercially available protein assay kit following the manufacturer's instructions and using 96-well flat-bottom plates and a microplate reader. Determine the average of these three values.

   In the authors' experience, the S9 protein content of pooled samples averages ∼26 mg/ml (CV ≈ 5%). Variability among samples from a single pooled homogenate is generally low (CV <2%), although each laboratory using the protocol for the first time should confirm this. With experience it may be possible to reduce the amount of sample replication recommended here. Sample remaining after performing this step should not be re-frozen for use in metabolic stability assays. In general, each sample is thawed and used once. The one exception is that thawed samples may be refrozen for use as heat-denatured control material.

Pre-prepare reagents

2. On the day before the experiment, prepare an adequate volume of solution C and adjust the pH to 7.8.

   The required volume of solution C can be estimated as 190 μl times the number of reaction vessels in an experiment, plus an additional volume to account for pipetting losses and make up reaction cofactors. For the experiment shown in Figure 14.10.6, the volume totals ∼11 ml. If several experiments are to be run within a short period of time, however, it is recommended that a larger volume be made up (e.g., 100 ml) and the excess stored for future use. If stored solution C is already available, the pH should be checked the day before running an experiment.

3. Weigh out the required amounts of NADPH (solution F), UDPGA (solution G), GSH (solution H), and PAPS (solution I), and transfer these cofactors to labeled 2-ml test tubes or plastic vials. Seal tubes or vials with Parafilm and store at −20°C.

   At this point, only weigh out the dry stocks necessary for the anticipated (calculated) volume of each respective reagent required.

4. Prepare a stock solution of test compound in an appropriate solvent.

   The solvent utilized will depend on the nature and solubility of the test compound. Pure water should be used if possible. Stock solutions should be refrigerated when not being used. Some stock solutions may be made up well in advance of an experiment. Under these circumstances, however, the stability of the test compound should be evaluated. If the test compound is light sensitive, store in a brown/amber container.

5. If an organic solvent is used to create the stock solution, dilute the stock solution with solution C to create the desired spiking solution.

   Because organic solvents may depress enzyme activity, the concentration of solvent in the final reaction mixture should be kept to a minimum. One way to minimize the amount of organic solvent in the mixture is to dilute the test chemical stock solution with solution C before adding it to the system. This approach also increases the volume of spiking solution added to the mixture, improving accuracy and precision in pipetting. As an example, the stock solution may be diluted 1:5 with solution C to create a spiking solution containing 20% organic solvent. The addition of 10 μl of this spiking solution to the reaction system (final volume of 200 μl) will result in 1% organic solvent content. Solubility tests should be performed beforehand in the spiking solution (if different from the stock solution), and the solution should be refrigerated when not being used. Caution is advised to ensure that the chemical does not precipitate under these conditions.

   Subsequent volume recommendations pertaining to the stock solution of test compound are based on an assumption of a 20× working stock. This is true whether the stock of test compound is made according to directions in step 4 only, or whether step 5 was performed as well. Lower concentrations of working test compound stocks (e.g., 10×) may be prepared if necessary (i.e., to facilitate solubility), but if this is the case, the volume of test compound stock to add to start the experiment—step 25 must be adjusted accordingly; likewise, the volume of solution C required to bring solutions D1 (step 15) and D2 (step 16) to the desired target should be adjusted.

   In all cases, the overall organic solvent percentage in the incubation mixture must not exceed 1%.

   See supplemental material: CPTX_14.10_Supplemental_Tool.xisx. (http://www.currentprotocols.com/protocol/tx1410). This spreadsheet is a quick calculation tool that is pre-populated with values specified within Basic Protocol 2, and automatically adjusts master mix volumes according to permissible concentration ranges and experimental conditions. The spreadsheet contains instructions for use, and generates two quick-reference tables that are meant to be printed out for use at the bench.

   Note that the supplemental calculation tool has an additional feature for calculating volume requirements for standards; discussion of standards is beyond the scope of this

unit, as quantification methods and requirements vary considerably from laboratory-to-laboratory, and even across experiments.

Perform experiment

6. Turn on the shaking water bath and circulating chiller, and adjust the temperature control on the chiller to 12°C.

7. Set up the test tubes: use glass 96 deep-well format (with rack) or amber glass test tubes (Target DP T/S vials) necessary for an experiment in a format such as that given in Figure 14.10.7. Arrange the tubes in an appropriate rack or sample holder and place on ice.

   Figure 14.10.7 shows a basic experiment designed to measure substrate depletion at one test chemical concentration. With experience, it is possible to run more complex experiments involving, e.g., use of two different S9 samples or two different test chemicals. Two different test chemical concentrations also may be tested; however, preliminary experiments are generally performed to determine the optimal concentration for a given test chemical (see Commentary).

8. Calculate the number of active and heat-denatured samples that comprise an experiment and increase each number by 25% to 30% to provide a modest excess of biological material.

   For example, the experiment shown in Figure 14.10.7 includes 18 active samples (three replicates × six time points); adding five more samples (to provide excess material) gives a total of 23. A similar calculation is done for the heat-denatured samples, (three replicates × three time points) plus three additional gives a total of 12.

9. Calculate the required total volumes of solutions E, F, G, H, and I, assuming 20 μl for each reaction vessel.

   For example, multiplying 20 μl by the number of active samples (i.e., 23) gives a total volume of 0.460 ml, or 460 μl. An additional 240 μl (20 μl times 12) would be required for heat-denatured samples. A total volume of 700 μl would be required. Therefore, 1 ml of each solution is sufficient to conduct an experiment.

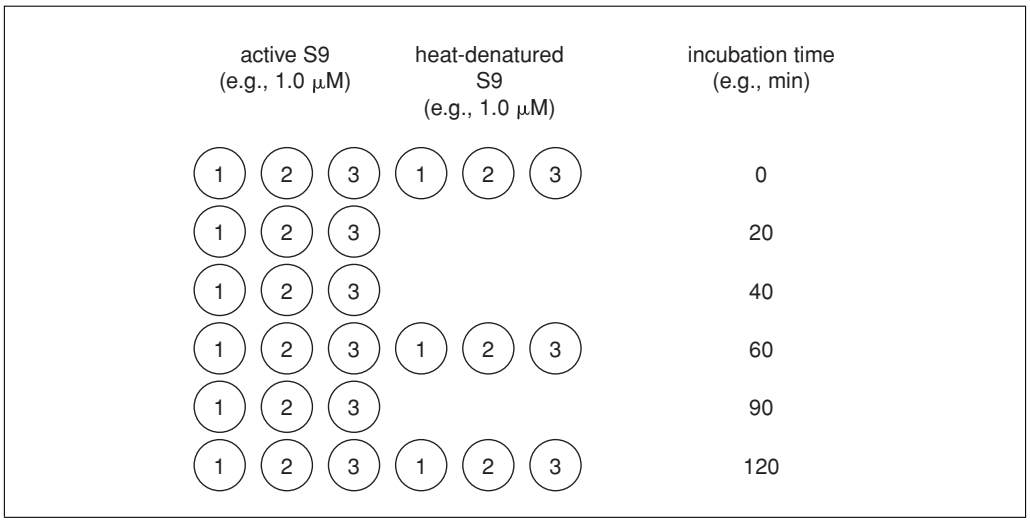

Figure 14.10.7 Rainbow trout S9 fraction incubation layout. The experiment pictured is designed to measure substrate depletion at one test chemical concentration (1.0 μM) over a period of 120 min. Active samples consist of active S9 protein in 100 mM potassium phosphate buffer containing alamethicin, cofactors, and test chemical. Heat-denatured samples are identical to active samples except that the S9 protein has been inactivated by heat denaturation. Heat-denatured controls are sampled at 0 min, the mid-point of the experiment (e.g., 60 min), and the longest incubation time (e.g., 120 min).

10. Calculate the required total volume of solution D1 (active S9 fraction), assuming 90 µl for each reaction vessel. For example, multiplying 90 µl by 23 gives a total volume of 2.070 ml.

    The assumption of 90 µl is based on a final reaction volume of 200 µl, a 20× concentrated test compound stock (recall that if test compound stock is made in organic solvent, the concentration of solvent must never exceed 1%, relative to final reaction volume), and concentrations of solutions E, F, G, H, and I as specified in Reagents and Solutions. The delta between stock protein concentration and desired assay protein concentration is assumed to be in a reasonably high range (using the conditions listed in this protocol, the stock protein concentration should be at least five times greater than the desired assay protein concentration, and preferably at least ten times greater).

    Solutions E, F, G, H, and I are made up in solution C; therefore, treat the volume calculation for solution C in this step as one would for water or a similar neutral buffer in a typical master mix, i.e., increase or decrease the volume of solution C for this step to ensure the accuracy of the final concentrations of the other assay components.

11. Calculate the volume of active S9 fraction that must be added to solution D1 based on the average measured protein content from step 1.

    To determine the required volume of S9 fraction, multiply the desired protein content in the final reaction mixture (mg/ml) times the final reaction volume (ml), and divide by the measured protein content of the S9 fraction (mg/ml). For example, assuming a desired final protein content of 2 mg/ml, 0.2 ml reaction volume, and 25 mg/ml protein content of the S9 sample, the required volume of S9 is (2 × 0.2)/25, or 0.016 ml (equivalent to 16 µl). Multiplying this value by 23 gives 368 µl, which is the total volume of active S9 fraction required to create solution D1.

12. Repeat steps 10 and 11 to calculate the required total volume of solution D2 (heat-denatured S9 fraction) and the volume of added S9 fraction (heat-denatured).

    It is assumed that heat-treatment does not change the protein content of the sample.

13. Partially thaw the required volumes of active and heat-denatured S9 fraction, and store on ice until used.

    Based on the calculation given in step 11, it is apparent that the experiment shown in Figure 14.10.7 can be run using one, 0.5-ml sample of active S9 fraction and a second, 0.5-ml sample of heat-denatured material.

14. Prepare solution E and store on ice until use.

15. Pipet active S9 fraction into a 25-ml Erlenmeyer flask containing ice-cold solution C to create solution D1. Vortex the solution briefly and place on ice.

    The required volume of solution C is determined by subtracting the added volume of S9 fraction (from step 11) from the total volume of D1 calculated in step 10. In this example, the required volume of solution C is 2.070 ml minus 368 µl, or 1.702 ml.

16. Repeat step 15 using a heat-denatured S9 fraction to create solution D2.

17. Pipet the required total volume of solution E into solution D1. Incubate this solution for 15 min on ice.

    The addition of alamethicin to the incubation system is based on its standard use in mammalian depletion assays. Additional research is needed to determine whether or not alamethicin should be used or under what specific circumstances this solution is needed to support in vitro assays with fish.

18. Repeat step 17 for solution D2.

19. While the solutions created in steps 17 and 18 are incubating, prepare cofactor solutions F, G, H, and I.

20. Pipet the required total volumes of solutions F, G, H, and I into solution D1 and vortex briefly. Transfer this master mix to a chilled 250-ml beaker to facilitate pipetting.

21. Repeat step 20 for solution D2.

22. Using a repeater pipettor, pipet 190 µl of solution D1 into each of the active S9 reaction vessels.

23. Repeat step 22 for solution D2.

24. Transfer the entire sample set to the water bath and equilibrate for 10 min with shaking at 12°C.

25. Using a repeater pipettor, dispense 10 µl of spiking solution containing the test chemical into all test tubes (D1 and D2) to initiate the reaction. If the chemical under investigation is known to be highly volatile, cover the test tubes loosely with a non-sterile 96-well cap mat to minimize chemical losses.

    It is important that T = 0 min samples be treated differently from other samples. Because some compounds may be rapidly metabolized (i.e., esters), it is recommended to add the stopping solution to tubes containing the master mix before adding the test chemical of interest; this ensures that the reaction does not start before the stopping solution is added. Also, allow a fixed length of time (e.g., 15 sec) to elapse before adding chemical to the next row of tubes (corresponding to the next reaction time point). This time delay is then taken into account when the reaction is stopped (step 26), ensuring that actual incubation times are as close to planned incubation times as possible. A timer and notepad may be useful to manage the addition of test chemical and stopping solution.

    The recommended volume of 10 µl here assumes a 20× concentrated test compound stock. For less concentrated stocks, dilute to match a setting on the repeater pipettor to facilitate speed (e.g., if the next highest step after 10 µl on the repeater pipettor is 25 µl and the concentration of the test compound stock is 10×, dilute the stock to 8× to use the 25-µl setting, rather than pipetting 10 µl of a 10× concentrated stock two times).

    The concentration of organic solvent, if used in preparation of the stock compound, must never exceed 1% relative to the final reaction volume.

26. Using a repeater pipettor, stop the reaction at selected time points by adding 1 to 3 vol (200 to 600 µl) of organic solvent appropriate for the selected analytical method. Vortex each tube immediately after adding the solvent and place on ice until all incubates have been collected.

    The volume of organic solvent utilized for compound extraction is determined during analytical method development. When stopping the reaction, it is necessary to account for the time lapse that exists between samples (due to the finite length of time required to pipet in the test compound). Use of a laboratory timer may help facilitate this process.

27. Analyze samples using a validated method.

    All samples should be thoroughly vortexed (a multi-tube vortex may be useful for this step) although a standard vortex is recommended for time-critical steps and centrifuged for extraction. A subsample of the supernatant may then be transferred to a sealed test tube or vial. For most applications, samples that are not analyzed on the same day may be stored at −20°C. Storage conditions should be validated in advance.

28. After the samples are analyzed, log-transform measured concentrations of parent chemical and plot as a function of time. If the reaction exhibits first-order kinetics, the data will describe a straight line and may be analyzed by simple linear regression using all measured values (Fig. 14.10.8). Multiply the fitted slope term from this regression equation by −2.3 to obtain the first-order depletion rate constant (k_e), which has units of inverse time (e.g., 1/min).

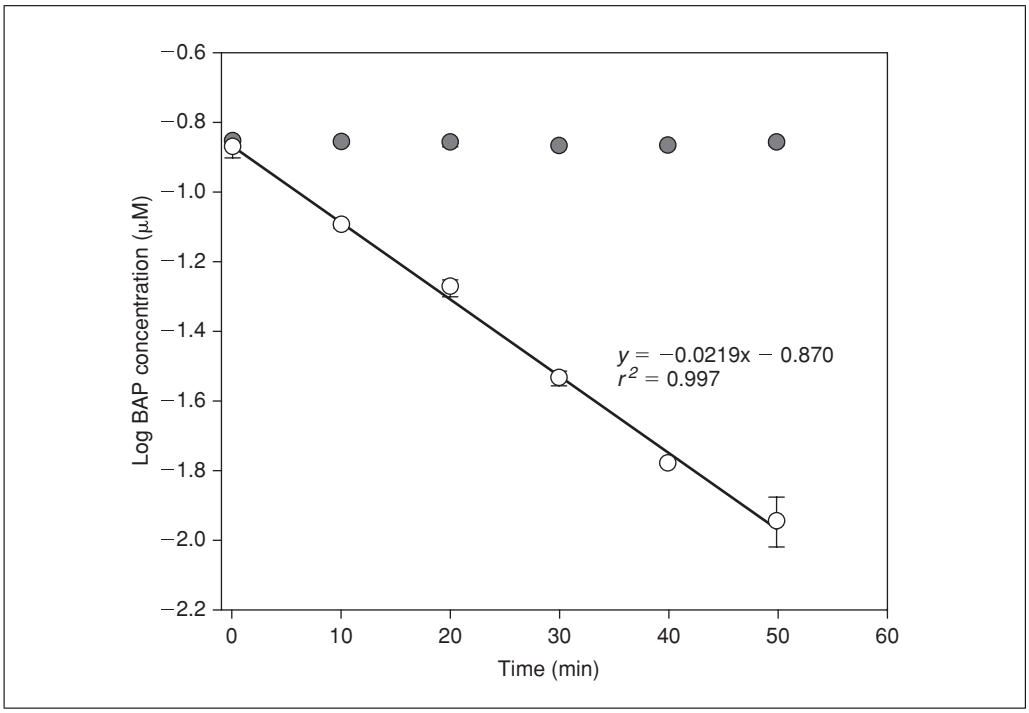

Figure 14.10.8 Representative plot of substrate depletion data. Measured concentrations of benzo[a]pyrene (BAP) at each time point are plotted as log-transformed values and used to develop a linear regression. Each point represents the mean (±SD) of three replicate determinations. The slope of this line is used to calculate a first-order depletion constant (k_e), which has units of inverse time (e.g., 1/min; see Basic Protocol 2, step 28). Note that chemical concentrations in heat-denatured control samples do not change over time and that starting concentrations for both sample types are essentially the same.

Deviations from first-order kinetic behavior can occur and may be due to several factors including saturation of metabolic activity, loss of enzyme activity over time, and product inhibition. These and other factors are discussed in the Commentary. In general, using the lowest possible substrate concentration is recommended, keeping in mind the need to measure concentrations well below the starting level. Reducing the S9 protein content will often result in improved system performance but must be balanced against the need for measureable activity.

29. Multiply the depletion rate constant (k_e) by the volume of the incubation system (e.g., 200 μl or 0.2 ml), divide by the measured amount of protein (e.g., 0.2 mg), and multiply by 60 (to convert minutes to hours) to calculate in vitro intrinsic clearance ($CL_{int,\ in\ vitro}$), which has units of ml/hr/mg protein (Equation 14.10.1).

$$CL_{int,\ in\ vitro} = \left[\left(\frac{k_e * \text{volume of incubation system}}{\text{quantity protein}}\right) * 60\right]$$

Equation 14.10.1

Multiply $CL_{int,\ in\ vitro}$ by the concentration of S9 protein per gram of liver and liver weight as a fraction of total body weight (the HSI, from Basic Protocol 1, step 15) to calculate in vivo intrinsic clearance ($CL_{int,\ in\ vivo}$), which has units of ml/hr/g fish. Equation 14.10.2 presents this in simplified terms, while Equation 14.10.3 provides a detailed version of Equation 14.10.2 with individual component calculations.

$$CL_{int,\ in\ vivo} = CL_{int,\ in\ vitro} * \left(\frac{\text{concentration of S9 protein}}{\text{gram liver}} \right) * HSl$$

Equation 14.10.2

The concentration of S9 protein per gram of liver is calculated as the protein content of the S9 fraction times the total volume of S9 fraction obtained from a single sampling run, divided by the total weight of livers processed (Equation 14.10.3). This calculation assumes that recovery of metabolizing enzymes from the liver homogenate (i.e., in the S9 supernatant) is 100% efficient. An additional correction term may be required if recovery is <100% efficient. Using glucose-6-phosphatase (G6P) as a marker for microsomal enzyme activity, Shultz and Hayton (1999) determined that recovery of microsomal activity in liver S9 fractions from several fish species ranged from 35% to 60%. To incorporate this correction, the $CL_{int,\ in\ vivo}$ determined above would have to be divided by the fractional recovery of G6P activity (e.g., 0.5). Additional research is needed to determine whether this correction should be applied on a routine basis.

$$CL_{int,\ in\ vivo} = CL_{int,\ in\ vitro} * \left[\left(\frac{\text{protein content of S9 fraction*total volume of S9 fraction}}{\text{total weight of livers processed}} \right) * \left(\frac{\text{liver weight}}{\text{total body weight}} \right) \right]$$

Equation 14.10.3

HEAT-DENATURED OR INACTIVE RAINBOW TROUT LIVER S9 FRACTION PREPARATION

SUPPORT PROTOCOL

Rainbow trout S9 fractions are denatured to provide negative control material for the substrate depletion assay. Additional denatured material may be used for constructing analytical standard curves. Protein denaturation is achieved by heating 10 min at 100°C (in boiling water) in a capped vial. The resulting sample is then processed using a hand-held homogenizer to produce a sample that can be easily pipetted.

Materials

Rainbow trout liver S9 fraction with known protein content
Glass container or 16 × 100-mm borosilicate tube with caps
250-ml glass beaker
Hot plate or Bunsen burner
Floating test tubes racks (e.g., Fisher, cat no. 14-127-45)
15-ml Wheaton Tenbroeck hand-held tissue homogenizer (VWR, cat. no. 62400-530)
1.8-ml working volume cryogenic storage tubes (e.g., Thermo Scientific Nunc; Cole-Palmer, cat. no. EW-03755-10)

1. Thaw 5 ml of S9 fraction and place on ice.
2. Transfer the S9 fraction to a glass container or 16 × 100-mm borosilicate test tube and loosely cap to avoid evaporation during heat treatment (which would change the protein concentration).

   Make sure the cap is tightened loosely to avoid pressure build up and possible rupture of the container.

3. Boil water in a 250-ml beaker on a hotplate. Once the water is boiling, introduce the loosely capped container or test tube containing the S9 fraction on a floating test rack. Boil for 10 min.

4. Allow the heat-denatured S9 solution to cool until warm and transfer to a hand-held 15-ml homogenizer. Homogenize the sample by hand until the solution is homogeneous.

   The boiled S9 fraction is somewhat gelatinous. To facilitate its homogenization, the sample volume must be matched to the volume of the homogenizer (i.e., sufficient space must exist in the homogenizer to permit efficient homogenization without losing sample during the process). A sample volume appropriate for use with a 15-ml homogenizer (e.g., 5 ml) is recommended. These volumes (S9 sample and homogenizer) may be scaled up to process larger amounts of material.

5. Dispense 0.5 ml of heat-denatured S9 fraction into 1.8-ml cryogenic storage tubes and store up to 2 years at −80°C until used.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Refer to the Certificate of analysis (CoA) and MSDS for each test chemical and reagent to determine appropriate storage and handling conditions. The purity of the test compound is critically important and should be taken into account when calculating the mass needed to create stock and spiking solutions. Reagent purity values stated below are based on those given by suppliers of products listed in the Materials sections. With the exception of PAPS, it is not necessary to account for purity values <100% since actual purity values generally exceed 90%. The purity of PAPS is typically ∼80%, but may vary from 75% to 85%. The mass of PAPS needed to create a 1 mM solution should therefore be adjusted based on the reported purity of the material.

All reaction co-factors are provided in substantial excess of amounts needed to support Phase I and II metabolic pathways. Stated molar concentration values are approximate. If, as recommended, these solutions are made by dissolving pre-weighed amounts of material in a fixed volume of solution C (1 ml, introduced by pipet), actual molar concentrations will be slightly lower. Recommended reagent volumes reflect differences in how each solution is used. Solutions that can be stored between uses are made up in amounts that will support several experiments. Solutions that are created fresh before each use are made up in amounts that will support the substrate depletion experiment shown in Figure 14.10.7. These amounts must be adjusted if the intent is to run a more complex experiment.

Alamethicin in methanol and phosphate buffer (solution E), 250 μg/ml

Add 0.1 ml of methanol to this vial per milligram of alamethicin (purity ≥98%, assume 100% if ≥90%; in amber glass; Sigma Chemical Co., cat. no. A4665) creating a 10 mg/ml solution. Replace the cap, mix by inversion, and seal with Parafilm. This solution may be stored at −20°C until used. Solution E is created by pipetting 25 μl of the methanol/alamethicin solution into a plastic test tube or vial, and then adding 0.975 ml of ice-cold solution C (see recipe). Vortex until the chemical is in solution and cloudiness disappears. Prepare 1 day prior to the experiment day and store at −20°C. Alternatively, prepare fresh the day of the experiment and store on ice before use.

In principle, it is possible to add 0.250 mg alamethicin to a 1-ml volumetric flask, add 25 μl of methanol, and then make up to volume with solution C. In practice, however, it is difficult to measure out this small mass of material, given the amount that is typically purchased (e.g., 10 mg).

Clearing buffer, pH 7.8 (solution A)

Supplement Hank's balanced salt solution (HBSS without Ca^{2+} and Mg^{2+}; GIBCO/Life Technologies, cat. no. 14175-095) with 4.2 mM sodium bicarbonate ($NaHCO_3$; 0.35 g/liter; Sigma Chemical Co., cat. no. 56014), then add 0.5 M ethylenediaminetetraacetic acid (EDTA, dibasic; GIBCO/Life Technologies, cat. no. 15575-038) (target concentration 2.3 mM) and 1 M HEPES (MP Biomedicals, Fisher Scientific, cat. no. 1688449) (target concentration 10 mM). For example: to 985.4 ml of HBSS (containing $NaHCO_3$), add 4.6 ml of 0.5 M EDTA and 10 ml of 1 M HEPES. Adjust pH to 7.8 using 10 mM NaOH (see recipe) and store at 4°C. Prepare fresh monthly and discard if visibly contaminated.

DL-Dithiothreitol (DTT), 100 mM

Transfer 154.25 mg of DL-dithiothreitol (DTT; Sigma Chemical Co., cat. no. D9163; mol. wt. = 154.25 g/mol; purity ≥99%, assume 100% if ≥90%) to a 10-ml volumetric flask and bring up to volume with ultrapure water. Store at 4°C. Prepare fresh monthly and discard if visibly contaminated.

GSH (solution H), ∼5 mM

Transfer 1.536 mg of L-glutathione (GSH; solution H; Sigma Chemical Co., cat. no. G6529; mol. wt. = 307.2 g/mol; purity ≥98%, assume 100% if ≥90%) to a 2-ml test tube or plastic vial. Dissolve by pipetting in 1 ml 100 mM ice-cold solution C (see recipe) and vortex briefly. Prepare 1 day prior to the experiment day and store at −20°C. Alternatively, prepare fresh the day of the experiment and store on ice before use.

Homogenization buffer (solution B)

Dilute 1 M Tris·Cl (see recipe) to 50 mM by combining 50 ml of 1 M Tris·Cl and 950 ml of ultrapure water. In a 1-liter volumetric flask, combine ∼800 ml of 50 mM Tris·Cl with 150 ml of 1 M potassium chloride (see recipe), 4 ml of 0.5 M EDTA (dibasic; GIBCO/Life Technologies, cat. no. 15575-038), 10 ml of 100 mM DTT (Sigma Chemical Co., cat. no. D9163), and 85.55 g sucrose (mol. wt. = 342.2 g/mol; purity ≥99.5%, assume 100% if ≥90%; Sigma Chemical Co., cat. no. S7903). Adjust pH to 7.8 with 1 M potassium hydroxide (KOH; see recipe) and then bring up to final volume with 50 mM Tris. Store at 4°C. Prepare fresh monthly and discard if visibly contaminated.

NADPH tetrasodium salt (solution F), ∼20 mM

Transfer 17.6 mg of nicotinamide adenine dinucleotide 2′-phosphate, tetrasodium salt (NADPH; solution F; mol. wt. = 833.4; Oriental Yeast Co., cat. no. 44332900) to a 2-ml test tube or plastic vial. Dissolve by pipetting in 1 ml ice-cold solution C (see recipe) and vortex briefly. Prepare 1 day prior to the experiment day and store at −20°C. Alternatively, prepare fresh the day of the experiment and store on ice before use.

Solutions F, G, H, and I are prepared by weighing out cofactors the day before an experiment. Each cofactor is transferred to a test tube or vial, which is then sealed with Parafilm and stored overnight at −20°C.

PAPS (solution I), ∼1 mM

Transfer 0.634 mg of 3′-phosphoadenosine 5′-phosphosulfate (PAPS; mol. wt. = 507.26 g/mol; reported purity is typically ∼80% but may range from 75% to 85%; solution I; Sigma Chemical Co., cat. no. A1651) to a 2-ml test tube or plastic vial.

continued

Dissolve by pipetting in 1 ml ice-cold solution C (see recipe) and vortex briefly. Prepare by weighing out the amount and sealing with Parafilm 1 day prior to the experiment day and store at −20°C. Alternatively, prepare fresh the day of the experiment and use immediately to avoid lower efficiency.

Potassium chloride (KCl), 1 M

Transfer 7.455 g of potassium chloride (KCl; mol. wt. = 74.55 g/mol; purity ≥99%, assume 100% if ≥90%; Sigma Chemical, cat. no. P9541) to a 100-ml volumetric flask and bring up to volume with ultrapure water. Store at 4°C. Prepare fresh monthly and discard if visibly contaminated.

Potassium hydroxide (KOH), 1 M

Transfer 5.611 g of potassium hydroxide (KOH; mol. wt. = 56.11 g/mol; purity = 90%, assume 100% if ≥90%; Sigma Chemical, cat. no. 484016) to a 100-ml volumetric flask and bring up to volume with ultrapure water. Store at 4°C. Prepare fresh monthly and discard if visibly contaminated.

Potassium phosphate buffer, pH 7.8 (solution C), 100 mM

Mix together 100 mM potassium phosphate dibasic (see recipe) and 100 mM potassium phosphate monobasic (see recipe) to achieve a pH of 7.8. For example, to create ∼100 ml of buffer, combine 88 ml of potassium phosphate dibasic with 12 ml of potassium phosphate monobasic. Add additional potassium phosphate dibasic (base) or potassium phosphate monobasic (acid) to adjust the pH. Store at 4°C. Prepare fresh monthly and adjust pH as necessary before each use. Discard if visibly contaminated.

Potassium phosphate dibasic buffer, 100 mM

Transfer 1.742 g potassium phosphate dibasic (mol wt. = 174.2 g/mol; purity ≥99%, assume 100% if ≥90%; Sigma Chemical Co., cat no. 60353) to a 100-ml volumetric flask and make up to volume with ultrapure water. Store at 4°C. Prepare fresh monthly and discard if visibly contaminated.

Potassium phosphate monobasic buffer, 100 mM

Transfer 1.361 g potassium phosphate monobasic (mol. wt. = 136.09 g/mol; purity ≥99%, assume 100% if ≥90%; Sigma Chemical Co., cat. no. P5655) to a 100-ml volumetric flask and make up to volume with ultrapure water. Store at 4°C. Prepare fresh monthly and discard if visibly contaminated.

Sodium hydroxide, 10 mM

Pipet 1.0 ml of 1 M NaOH solution into a 100-ml volumetric flask and bring up to volume with ultrapure water. Store at 4°C. Prepare fresh monthly and discard if visibly contaminated.

Test chemical stock and spiking solutions

Prepare a stock solution by transferring an appropriate amount of test compound to a 1-ml volumetric flask. Make up to volume with the selected solvent. This stock may be used as a spiking solution if the selected solvent is water. If an organic solvent is used, the spiking solution may be prepared by pipetting 0.2 ml of stock solution into a second, 1-ml volumetric flask. Make up to volume with solution C. The test chemical concentration in the spiking solution should be designed so that addition of 10 µl to each reaction vessel (see Basic Protocol 2, step 25) results in the desired final concentration.

Tricaine methanesulphonate (MS 222), 300 mg/liter

Fill a 10-liter plastic bucket with 6 liters of water drawn from the same source as that used to maintain the fish. Add 1.8 g of tricaine methanesulphonate (MS-222; Argent Chemical Laboratories, cat. no. C-FINQ-UE) along with a mass of $NaHCO_3$ determined in advance to result in maintenance of source water pH. For low-alkalinity water, the required mass of $NaHCO_3$ is about three times that of MS-222.

Tris·Cl, 1 M (pH 7.8)

Transfer 157.60 g of Tris·Cl (Sigma Chemical, cat. no. T5941) to a 1-liter volumetric flask and bring up to volume with ultrapure water. Adjust pH to 7.8 using 10 mM NaOH and store at 4°C. Prepare fresh monthly and discard if visibly contaminated.

UDPGA (solution G), ~20 mM

Transfer 12.95 mg of uridine 5′-diphosphoglucuronic acid, trisodium salt (UDPGA; solution G; mol. wt. = 646.23 g/mol; purity = 98% to 100%, assume 100% if ≥90%; Sigma Chemical Co., cat. no. U6751) to a 2-ml test tube or plastic vial. Dissolve by pipetting in 1 ml ice-cold solution C (see recipe) and vortex briefly. Prepare 1 day prior to the experiment day and store at −20°C. Alternatively, prepare fresh the day of the experiment and store on ice before use.

COMMENTARY

Background Information

In fish as in mammals, the liver is the principal organ of chemical biotransformation. It is reasonable, therefore, to evaluate whole-animal metabolism using in vitro systems derived from liver tissue. This unit provides a protocol to assess metabolic stability of xenobiotics in rainbow trout using the liver S9 fraction, which is relatively easy to prepare and contains all of the enzymes responsible for Phase I and II metabolic reactions. The protocol employs a substrate depletion approach to measure intrinsic clearance under linear (first-order) reaction conditions. Implied by this approach is the assumption that metabolic pathways in fish generally operate under non-saturating conditions; that is, environmental exposures are not expected to result in circulating chemical concentrations that approach saturating levels. If the actual chemical concentration saturates pathways responsible for metabolism, then the rate of clearance measured using this protocol will tend to overestimate true levels of in vivo activity.

In contrast to assays that measure the appearance of one or more metabolic products, the substrate depletion approach provides no information on products or the identities of reaction pathways. Therefore, the protocol as described here cannot be used to investigate reaction types and mechanisms, including the induction, inhibition, and substrate specificity of different enzymes (e.g., CYP enzymes). It is well suited, however, to estimating in vitro and (by extrapolation) in vivo intrinsic clearance. Rainbow trout have been used extensively to study chemical metabolism by fish and for ecotoxicological research. The existence of information regarding trout biology and physiology also facilitates incorporation of metabolism information into computational models of chemical uptake and accumulation. Trout are frequently used to evaluate chemical accumulation by fish, and published accumulation data are available for many well-known environmental contaminants (Arnot et al., 2008).

To satisfy the requirement for first-order reaction conditions, substrate depletion assays are generally performed using starting chemical concentrations of ≤1 μM. This requires analytical methods possessing a high level of sensitivity and precision. While high-performance liquid chromatography (HPLC) may be sufficient to analyze some compounds, mass spectrometry methods such as gas chromatography-mass spectrometry (GC-MS) or liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) are likely to be required for many others. A review of analytical chemistry procedures is beyond the scope of this protocol; however, guidance on validation of the selected method is provided below. Additional guidance is given for selection and care of test animals, the design and interpretation of preliminary experiments, and use of appropriate experimental controls.

The methods given here are analogous to procedures used by the pharmaceutical industry to obtain screening-level metabolism information during preclinical stages of drug development (Iwatsubo et al., 1997; Obach et al., 1997). In practice, however, most drugs are ionized at physiological pH and/or possess relatively low (<4.0) log K_{OW} (or log $D_{7.4}$) values. By comparison, many environmental contaminants possess high (>4.0) log K_{OW} values, which promotes their accumulation by aquatic organisms. This hydrophobic behavior has implications for in vitro testing insofar as it promotes adsorption of test chemicals to reaction vessels and necessitates the use of organic solvents to dissolve chemicals into solution.

An important potential use of these methods is to predict metabolism impacts on chemical bioaccumulation by fish (Nichols et al., 2007, 2009). Computational models driven largely by log K_{OW}-based relationships accurately predict the accumulation of many compounds, provided that they do not undergo substantial biotransformation (Arnot and Gobas, 2003). Expert systems have been developed to predict likely metabolic products based on an evaluation of parent chemical structure (Mekenyan et al., 2005), and progress is being made toward predicting metabolic rate using structure-activity approaches (Arnot et al., 2009). Presently, however, metabolism represents the single greatest source of uncertainty in bioaccumulation modeling efforts. Although standardized in vivo testing procedures (OECD, 1996) can be used to directly assess chemical accumulation, these methods are time-consuming, costly, and utilize large numbers of test organisms.

Recently, several research groups have used in vitro metabolism data as an input to models of chemical bioaccumulation by fish (Han et al., 2007, 2008, 2009; Cowan-Ellsberry et al., 2008; Dyer et al., 2008; Gomez et al., 2010). The first step in this process is to predict in vivo hepatic clearance using a physiological model of the liver and appropriate in vitro-to-in vivo extrapolation factors. The estimated hepatic clearance is then incorporated into the bioaccumulation model using empirical algorithms that translate hepatic clearance into an estimate of whole-body clearance (Nichols et al., 2006). The success of these efforts has been evaluated by comparing measured levels of bioaccumulation in fish with modeled values. In all cases, modeled levels of accumulation that accounted for metabolism were much closer to measured values than were model predictions that assumed no metabolism. Collectively, these studies have provided an important "proof of concept" for the approach. Currently, however, a need exists to standardize these procedures so that results can be better compared among laboratories. Greater standardization also is required if these procedures are to become part of a generalized testing strategy to support bioaccumulation assessments for fish (Nichols et al., 2007, 2009).

Critical Parameters and Troubleshooting

Selection and treatment of research animals

Fish age and size recommendations are based on experience and represent a compromise between the need to obtain adequate amounts of tissue and a desire to avoid using animals that have achieved or are approaching sexual maturation. Trout held indoors may be maintained on a natural photoperiod or a photoperiod that has been manipulated to slow sexual maturation (e.g., extended use of a 16-hr light:8-hr dark, cycle). Extreme manipulations of photoperiod (e.g., permanent exposure to a 24-hr light:0-hr dark cycle) are not recommended as they may result in large individual differences in sexual development (Bourlier and Billard, 1984). The reproductive status of test animals may be assessed by measuring gonad weight and expressing this value as a gonadosomatic index (GSI), calculated as gonad weight divided by the total weight of the animal. It has been found that male and female trout of the recommended age and size possess relatively low GSI values (males <0.02, females <0.04) and exhibit little or no differences with respect to liver enzyme activities. Differences in metabolic activity between the sexes are expected to emerge as fish approach spawning conditions (Stegeman and Chevion, 1980; Hansson and Gustafsson, 1981; Förlin and Haux, 1990). Criteria for acceptance/rejection of test animals based on measured GSI values or the gross appearance of gonads have not been established. However, it is recommended that an effort be made to work with sexually immature animals. Manipulation of gender by hormone treatment, as commonly practiced in the aquaculture industry, is discouraged due to possible impacts on biotransformation enzymes. Similarly, care must be used when treating fish with various chemicals or drugs (as for disease abatement) since the effects of these treatments on metabolism are largely unknown. In general, such treatments should be avoided.

Recommended holding conditions for rainbow trout (e.g., water flow rate and holding density) are given by the American Society for Testing and Materials (ASTM, 2007).

Preliminary studies

Preliminary studies must be conducted to establish reaction conditions needed to reliably measure intrinsic in vitro hepatic clearance. The primary goal is to determine conditions that result in first-order depletion kinetics. These studies are also used to establish a sampling schedule that results in measureable depletion of test compound at all or most sampling times, as well as measureable levels of test chemical at later time points. As a general guidance, it is desirable to achieve 20% to 90% depletion of test chemical over the course of an experiment. Variables that can be manipulated to achieve this goal include the S9 protein content, the starting chemical concentration, and total incubation time.

Assuming that a validated analytical method has been developed, preliminary studies should be run at an S9 concentration of 1 or 2 mg/ml. The starting chemical concentration is determined by the need to achieve first-order kinetics as well as the sensitivity of the analytical method, keeping in mind the possible need to measure concentrations substantially lower than starting values (i.e., at later time points). The analytical method should be able to accurately measure 10% of the initial test substance concentration. Theory dictates that the likelihood of first-order kinetics increases as the starting concentration is decreased. Practical experience suggests that a starting concentration in the very low micromolar/high nanomolar range (e.g., 1.0 μM) often yields satisfactory results. This requires that the lower limit of quantitation (LLOQ) for the analytical method be <0.1 μM. All measured concentrations should lie within the defined range of the instrument calibration curve.

Preliminary studies are generally run using an intermediate (e.g., 1 hr) incubation time. Depending on the outcome, this can be adjusted from 15 min to 2 hr, recalling the need to incorporate six or more individual sampling times. If $>90\%$ depletion occurs in <15 min, it may be advisable to decrease the protein concentration. For hydrophobic organic chemicals, however, changes in S9 protein content are expected to result in changes in the free (unbound) chemical fraction in solution. Because metabolizing enzymes operate on the free chemical fraction, this means that a change in S9 protein content will tend to be offset by changes in free chemical concentration. Thus, an increase in S9 protein content will increase the number of enzyme molecules in solution but decrease the free chemical concentration available to be metabolized. The net result is that first-order elimination rate constants derived from these studies cannot be expected to vary in direct proportion to the S9 protein concentration. Very low rates of metabolism can be addressed by increasing the incubation time to 2 hr. Increasing the S9 protein concentration to promote increased levels of activity is not recommended due to increased non-specific chemical binding and possible product inhibition (see below).

A departure from first-order kinetics can be expected if the starting chemical concentration saturates the activity of enzymes responsible for chemical clearance. For reaction pathways that exhibit classical Michaelis-Menten kinetics, this saturation will result in zero-order elimination, which is indicated by linear depletion of measured chemical concentrations plotted on a numeric scale (i.e., prior to log-transformation). Log-transformation of the data will then result in a line that curves downward at later time points. The appearance of zero-order kinetics suggests that the starting chemical concentration should be reduced. Alternatively, log-transformation of the data may yield a pattern suggesting bi-exponential kinetics with an initial "fast" depletion phase followed by a "slow" terminal depletion phase. This pattern can be caused by product inhibition, wherein the accumulation of metabolic products inhibits enzymatic activity at later time points. Reduction of both the starting chemical concentration and S9 protein content may be attempted in an effort to eliminate this problem.

Positive controls

Each pooled S9 sample should be evaluated to determine its ability to catalyze Phase I and II metabolic reactions. By comparing this information to historical data and/or published information, the "representative" nature of a sample can be established, increasing confidence in the results of subsequent metabolic stability assays. In principle, this information also can be used to "benchmark" study results as a means of improving comparisons within and among laboratories.

A detailed description of standard metabolic assays is beyond the scope of this protocol. In general, however, these assays were first developed in studies with mammals, and their adoption for use with fish is based on

demonstrated conservation of the measured activity. Well-known assays for Phase I metabolic activity include measurement of testosterone hydroxylation (Smeets et al., 2002; Choi et al., 2005), lauric acid hydroxylation (Castle et al., 1995; Buhler et al., 1997), and aryl hydrocarbon hydroxylation (AHH; Van Cantfort et al., 1979). 1-Chloro-2,4-dinitrobenzene has been used extensively to measure Phase II glutathione S-transferase activity (Habig et al., 1974; Han et al., 2009). Standard substrates for measurement of Phase II UDP-glucuronosyltransferase (UGT) activity include p-nitrophenol (Hänninen, 1968) and estradiol (Mano et al., 2005).

At a minimum, three S9 samples should be characterized using at least one measure of Phase I activity and one measure of Phase II glucuronidation. Selection of a specific characterization assay (e.g., AHH) may be indicated if the same reaction pathway is thought to be responsible for clearance of selected test compounds (e.g., a group of polynuclear aromatic hydrocarbons). The amount of S9 material required to perform this characterization depends on the needs of the assays and desired level of replication. In most cases, these assays are run by pipetting S9 protein directly into a reaction mixture. The amount of S9 added to each tube (cuvette, etc.) is calculated from the measured protein content of the pooled S9 sample, the desired protein concentration in solution (e.g., 1 mg/ml), and the final volume of the reaction system.

Negative controls

Heat-denatured S9 samples are incorporated into each substrate depletion experiment to account for possible chemical losses due to abiotic degradation, volatilization, and adsorption to the reaction vessel. In the absence of any such losses, the measured chemical concentration will remain constant over time. A potential problem is indicated if the chemical concentration in heat-denatured samples declines by $>10\%$ during the course of an experiment. In some cases this loss can be addressed by making modifications to the assay system. For example, increasing the volume of the system, reducing its surface-to-volume ratio, may reduce volatility losses. In principle, data from negative controls can be used to correct measured levels of substrate depletion (e.g., by subtracting the slope developed from negative control data from that determined for active samples). This must be done with caution, however, keeping in mind the need to verify first-order reaction kinetics. For most applications, borosilicate glass vessels are preferred to vessels made of polymer plastic (Hirshmann glass inserts or amber glass GC vials are recommended). When working with hydrophobic compounds it is also important to minimize the number of sample transfers during analysis.

Matrix controls consisting of the complete S9 system (active protein, alamethicin, and all cofactors) without added chemical should be evaluated during analytical methods development to ensure that the system does not contribute chemicals that cause analytical interferences. Controls that contain active protein but no cofactors are often used in microsomal assays to detect non-CYP-mediated reactions. These controls are not recommended for the S9 system because the system already contains substantial levels of cofactors (even prior to their addition). Similarly, controls containing only alamethicin and cofactors are not recommended. Although these samples might in theory control for the effect of metabolic activity, chemical losses due to adsorption or volatility could be expected to differ from those occurring in a system that contained S9 protein.

Analytical methods

A validated analytical method should be developed for each test chemical. GC-MS is the method of choice for many semivolatile high log K_{ow} compounds. LC-MS/MS may be employed, provided that cationic components of the phosphate buffer do not interfere with the analysis. HPLC also may be used if the detection method (e.g., sample fluorescence) provides adequate sensitivity. If a laboratory performing the S9 incubations cannot analyze samples, extracted samples may be shipped to another facility. In this case, however, the stability of extracted samples must be evaluated under simulated shipping conditions. If samples are to be frozen prior to analysis, their stability under the expected storage conditions should be determined.

If an organic solvent is used to spike the chemical into the reaction system, solubility in this solvent should be determined. This solvent must be miscible with water to avoid the formation of a discrete partitioning phase within the reaction system. Potential spiking solvents include acetonitrile, acetone, methanol, and ethanol. The total amount of organic solvent in the reaction system should be kept at the lowest possible level to prevent deleterious effects on enzyme activity.

Each laboratory should determine acceptance criteria for nominal concentration values based on established Standard Operating

Procedures (SOP). The recovery of test chemicals from the reaction system must be determined and should range from 80% to 120%. Recoveries should be determined at the initial as well as 10% of the initial concentration to be sure the test substance behaves in a consistent manner across the concentration range of the assay. Standard or calibration curves are constructed by spiking test chemicals into heat-denatured S9 samples that contain all reaction cofactors. These samples are then processed in the same manner as other samples, including both the extraction and centrifugation steps. Calibration curves should be run before and after each sample set. These curves are then averaged to calculate nominal concentration values. Each standard curve should contain at least six concentrations. When comparing curves, the deviation among measured values for each standard should be $\leq$15% to 20% ($\leq$20% to 25% at the lower limit of quantitation, LLOQ). Standards may be prepared in advance and stored prior to use (typically at -20°C), provided that stability under these conditions has been confirmed.

An internal standard may be incorporated into the extraction solvent to evaluate sample analysis procedures. This standard also may be required to quantify the test compound (e.g., when using mass spectrometry methods). The internal standard should possess chemical characteristics closely resembling those of the test compound. For mass spectrometry, the deuterated form of the test chemical provides an ideal internal standard. Quality control (QC) samples representing high, medium, and low concentrations within the calibration curve should be incorporated into each sample run. These samples (three to four replicates per level) should be distributed throughout the sample set.

Pipetting technique

Experience suggests that successful implementation of these methods is critically dependent on the use of good pipetting technique. Accurate delivery of the chemical spiking solution (10 μl) into the reaction mixture is particularly important.

Anticipated Results

Five rainbow trout averaging 400 to 600 g will yield ~40 ml of liver S9 fraction possessing a protein content of ~26 mg/ml. These samples should be stored at -80°C. From the authors' experience, the inclusion of a stabilizer such as 250 mM sucrose will allow these samples to retain their initial activity for at least 2 years. If samples are stored for >2 years, repeating the initial characterization assays prior to their use to ensure maintenance of activity is recommended. The substrate depletion assay shown in Figure 14.10.7 will consume <0.5 ml of liver S9 fraction (i.e., one stored sample). Preliminary experiments designed to optimize the assay conditions may consume several times this amount. Analytical method development efforts (e.g., validation of chemical extraction methods) may utilize substantial quantities of S9 protein; however, most of this work is performed using heat-denatured material that has been stockpiled for this purpose. The researcher will encounter relatively greater challenges as the log K_{OW} value of test compounds increases due to decreased aqueous solubility (necessitating the use of an organic spiking solvent) and increased adsorption to reaction vessels. To date, however, a practical upper limit on the hydrophobicity of test compounds has not been established.

It is extremely important to validate analytical methods before attempting a substrate depletion experiment. Failure to do so may result in high variability among replicate determinations and/or nonsensical findings (e.g., a substrate depletion curve with a Y-intercept substantially different from the starting chemical concentration in heat-denatured samples). Based on the authors' experience, low S9 protein concentrations (e.g., 0.25 mg/ml) and short incubation times are needed to evaluate depletion of compounds such as esters that undergo rapid metabolism. In extreme cases, metabolism may proceed too rapidly to reliably measure. In such instances, however, this result can be used to establish a lower bound on the metabolism rate (i.e., the rate must be equal to or greater than an estimated value). Compounds that undergo measureable metabolism after 15 min to 2 hr can be evaluated using an S9 protein concentration of 1 to 2 mg/ml. Although it is preferable to deplete at least 20% of the starting chemical concentration during the course of an assay, the lowest metabolism rate that can be measured also depends on the level of agreement among replicate sample determinations at each time point. From a statistical point of view, the "sensitivity" of the method (i.e., ability to detect low rates of metabolism) depends on whether the log-linear depletion curve for active S9 samples differs significantly from the line described by heat-denatured controls. In some cases, it may be advantageous to include additional heat-denatured controls (i.e., at all

time points) as a means of better describing the behavior of these samples.

It is difficult to recommend firm criteria for acceptance or rejection of substrate depletion data because the challenges associated with each test chemical differ. Experience suggests, however, that the square of the correlation coefficient associated with fitted log-linear depletion curves (i.e., r^2) often exceeds 0.95. If the r^2 value for a particular experiment is <0.9, the data should be viewed with suspicion. The consistent appearance of r^2 values <0.9 suggests the existence of serious analytical problems and/or deviation from the assumption of first-order depletion kinetics. In either case, these problems should be addressed before conducting any more experiments. As the field advances, knowledge leading to improvements in S9 isolation methods, incubation conditions, and data analysis procedures will be gained. Ultimately, however, the goal is to generate information that can be confidently related to real levels of in vivo metabolic activity. To that end, efforts to improve in vitro testing procedures must be accompanied by research designed to measure and compare chemical metabolism rates in vitro and in vivo.

Time Considerations

Initially, it may be expected to take 3 to 6 months for a research team to become proficient in fish sampling, preparation of trout liver S9 fractions, S9 characterization (protein content and enzymatic activity toward standard substrates), and performance of substrate depletion assays. Once these skills have been mastered, the principal time limitation on performance of additional assays will be development and validation of analytical methods. This step may take days to weeks for each test compound depending on the experience of the researchers and availability of established methods. In some cases, a single analytical method may be adequate to measure several members of a chemical class, thus accelerating progress.

To process the recommended number of animals within an acceptable period of time, it is necessary to organize the S9 sampling effort as an "assembly line." A team of three researchers is recommended. One team member nets, anesthetizes, and weighs the animals, and is primarily responsible for record keeping. The other two are primarily responsible for perfusing livers, mincing tissue, and preparing tissue homogenates. All three team members contribute to snap-freezing the S9 fractions. This teamwork requires practice, and several dry runs may be needed before processing live animals.

Assuming the type of organization described above, upwards of 10 to 20 fish can be sampled and processed in 1 day. One or more days are required in advance of the fish sampling date to prepare buffers, stage dissecting equipment, and label sample tubes. In general, fish processing should be conducted in the morning to allow time for laboratory clean up. The time required to characterize a pooled S9 sample using standard measures of Phase I and II enzyme activity (i.e., positive controls) will depend on the number and complexity of these assays, but is likely to take several days or more. Performance of a substrate depletion assay based on the recommended experimental design (Fig. 14.10.7) may take 6 to 8 hr, spread over 2 days (3 to 4 hr of preparation the day before running an experiment and 3 to 4 hr to run the assay and prepare samples for chemical analysis). Depending on the individual sample run time (commonly 5 to 15 min), analysis of unknowns, calibration standards, and quality control samples (totaling ~48 separate injections) may require 4 to 12 hr. The time required to evaluate the resulting data, calculate chemical concentrations, plot the data, and calculate intrinsic hepatic clearance will depend on the experience of the researchers. These efforts may be greatly facilitated by the development of standardized spreadsheet templates for data entry and analysis.

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Disclaimer

For Marlies Halder: The views expressed are purely those of the author and may not under any circumstances be regarded as stating an official position of the European Commission.

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