Measurement of Soluble Epoxide Hydrolase (sEH) Activity

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ABSTRACT

The human soluble epoxide hydrolase (sEH; EC 3.3.3.2) is the product of the *EXPH2* gene. The sEH catalyzes the addition of a water molecule to an epoxide, resulting in the corresponding diol. Early work suggested a role of sEH in detoxifying a wide array of xenobiotic epoxides; however, recent findings clearly implicate the sEH in the regulation of blood pressure, pain, and inflammation through the hydrolysis of endogenous epoxy fatty acids such as epoxyeicosatrienoic acids (EETs). Both expression and activity of sEH are influenced by a wide array of xenobiotics, underlying how environmental contaminants could influence human health through sEH. This unit describes radiometric, fluorimetric, and mass spectrometric assays for measuring the activity of sEH and its inhibition. *Curr. Protoc. Toxicol.* 33:4.23.1-4.23.18. © 2007 by John Wiley & Sons, Inc.

Keywords: epoxyeicosatrienoic acids • blood pressure • inflammation • inhibition

INTRODUCTION

The human soluble epoxide hydrolase (sEH; EC 3.3.3.2) is the product of the *EXPH2* gene. The human sEH is a homodimer with each monomer comprised of two distinct structural domains linked by a proline-rich peptide (for a recent review see Newman et al., 2005). Interestingly, both domains have a catalytic activity: the epoxide hydrolase activity resides in the *C*-terminal domain, while the *N*-terminal domain catalytic site is a functional phosphatase. This unit concentrates on the epoxide hydrolase activity. The *C*-terminal domain of sEH catalyzes the addition of a water molecule to an epoxide, resulting in the corresponding diol (Morisseau and Hammock, 2005). Endogenous substrates for sEH include epoxy-fatty acids such as epoxyeicosatrienoic acids (EETs), which are known for their vasodilatory effects as well as for their anti-inflammatory actions (Newman et al., 2005). Hydrolysis of the epoxides by sEH diminishes this activity. The inhibition of sEH leads to the accumulation of EETs and other lipid epoxides in the organism. Furthermore, sEH inhibition in rodent models can successfully treat hypertension, pain, and inflammatory diseases, as well as protect against renal and vascular damages.

The sEH is mostly located in the peroxisomes and cytosol of cells in the liver and of many other organs. Cytosolic preparations, whole cultured cells, or purified sEH are used in enzymatic assays. The activity of sEH is measured as the moles of diol formed from substrates during a given time period via catalysis by a standard amount of protein. Three methods for measuring sEH activity are described in this unit: (1) a radioactive assay (Basic Protocol 1), which is very sensitive and can be used with various substrates (see Alternate Protocol 1); (2) a fluorimetric assay (Basic Protocol 2), which is very sensitive and useful for measuring inhibitory potency or for chemical library screening (see Alternate Protocol 2); and (3) an LC-MS/MS method (Basic Protocol 3), a general screening method, especially for natural substrates. The structures of the different substrates are given in Figure 4.23.1. Basic Protocols 1 and 3 can be performed with tissue extracts or purified enzyme, while Basic Protocol 2 requires the use of a purified or treated enzyme. Support Protocols 1 and 2 provide directions for synthesizing sEH substrates that are not commercially available.

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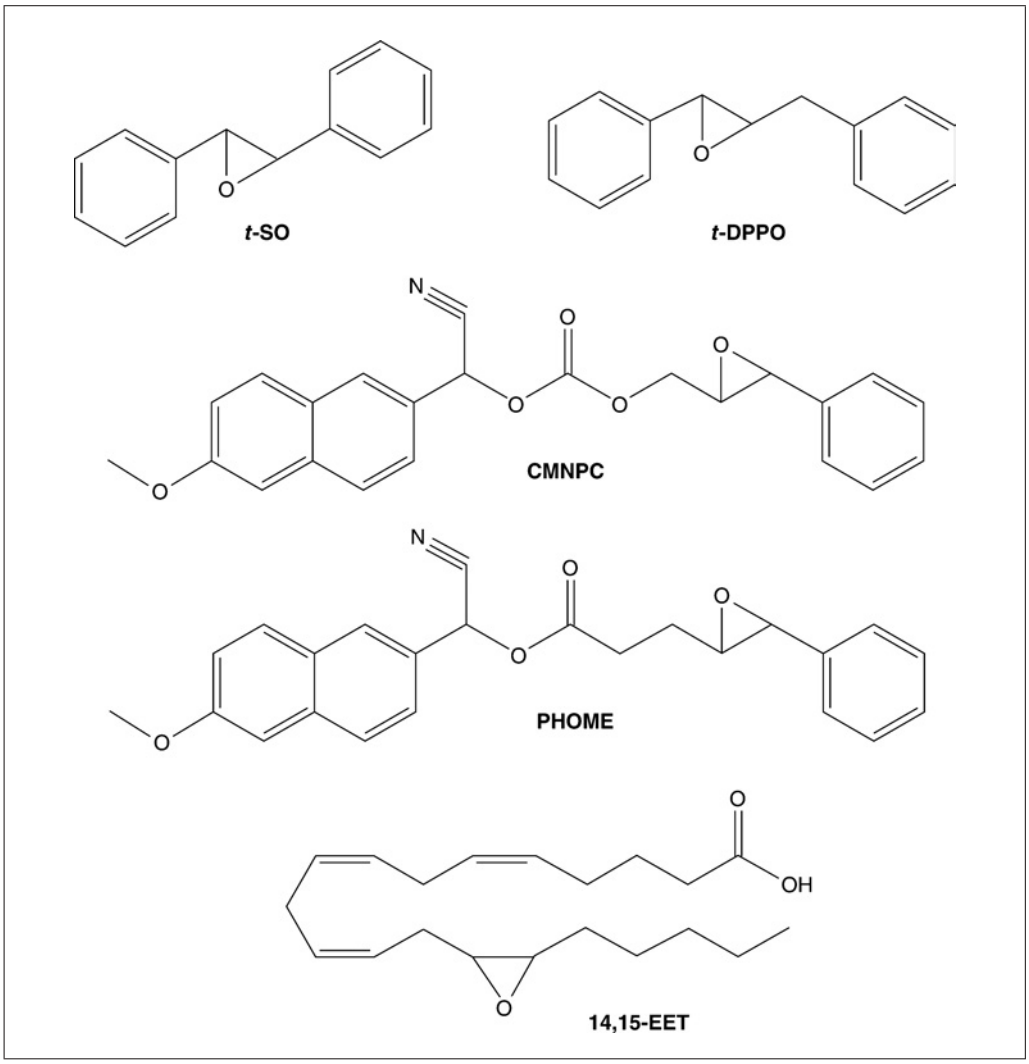

Figure 4.23.1 Substrates used in the protocols to measure soluble epoxide hydrolase (sEH) activity. Abbreviations: *t*-SO, *trans*-stilbene oxide; *t*-DPPO, *trans*-diphenyl-propene oxide; CMNPC, cyano(2-methoxynaphthalen-6-yl)methyl (3-phenyloxiran-2-yl)methyl carbonate; 14,15-EET, 14,15-epoxyeicosatrienoic acid; PHOME, (3-phenyl-oxiranyl)-acetic acid cyano-(6-methoxy-naphthalen-2-yl)-methyl ester.

When performing assays, it is wise to examine the original description of the method and subsequent comments on it. Wixtrom and Hammock (1985) have described some general procedures for the development and optimization of EH assays for different applications. There are additional valid methods in the literature that so far have found wide use. Any of these methods can, of course, be optimized to increase speed, sample throughput, and reproducibility and to decrease cost or alter many other parameters.

BASIC PROTOCOL 1

RADIOMETRIC ASSAY FOR sEH USING *trans*-STILBENE OXIDE (*t*-SO)

This protocol (Gill et al., 1983) describes a fast and accurate procedure for quantifying sEH in tissue extracts. The assay is based on differential partitioning between the epoxide substrate, the 1,2-diol product, and (if necessary) glutathione conjugates. *Trans*-stilbene oxide (*t*-SO) is also a substrate for glutathione-*S*-transferases (Gill et al., 1983); thus, if using a crude enzyme preparation, this parasitic activity must be taken into account. For this purpose, extraction with two solvents of different polarity was designed. The first solvent, isooctane, is a nonpolar solvent that extracts only the hydrophobic epoxide substrate out of the water phase and leaves the diol and glutathione conjugates in the buffer

hypophase. The second solvent used (hexanol) is more polar and allows extraction of both the epoxide and diol out of the water phase, leaving only the glutathione conjugates in the lower buffer phase. While this assay is versatile, it is not highly sensitive as presented (limit of detection of ~5 μg/ml of purified human sEH) because of relatively low turn over. The *t*-SO substrate can be labeled at high specific activity (Hammock et al., 1984); this, along with other modifications (e.g., longer incubation time), can dramatically increase the sensitivity of the assay. A method that is 100-fold more sensitive for measuring sEH is presented in Alternate Protocol 1. Following the alternate protocol, but substituting *cis*-stilbene oxide for *t*-SO and Tris·Cl buffer (0.1 M, pH 9.0) for the sodium phosphate buffer, will allow the quantification of microsomal EH in tissue extracts (Gill et al., 1983).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in the appropriately designated area, following the guidelines provided by the local radiation safety officer (also see *APPENDIX 1A*).

Materials

Tissue extracts (S-9 fraction, cytosolic fraction, or purified enzyme, treated, if necessary, to remove glutathione; see Wixtrom and Hammock, 1985)
0.1 M sodium phosphate buffer, pH 7.4 (*APPENDIX 2A*)/0.1 mg/ml fraction V BSA (Sigma), ice cold; add BSA just before use
5 mM [^{3}H]*trans*-stilbene oxide (*t*-SO) in ethanol containing ~10,000 cpm/μl (see recipe)
HPLC-grade isooctane and hexanol
95% (v/v) ethanol
Scintillation fluid

10 × 75–mm borosilicate glass tubes
Timer
30°C water bath
50-μl glass Hamilton syringe equipped with a repeating dispenser
Dispensers (0.1 to 1 ml) for organic solvents
50-μl glass Hamilton syringe with a blunted-end 22s-G needle
1.5-ml clear polypropylene microcentrifuge tubes
Liquid scintillation counter

Prepare test tubes

1. Place as many 10 × 75–mm borosilicate glass tubes in icy water as required to perform the assay:

   6 tubes for each tissue extract (or dilution of tissue extract) to be tested: 3 for isooctane extraction (Iso) and 3 for hexanol extraction (Hex) in steps 5 and 6
   3 tubes to determine total radioactivity (R) in the assay (not extracted)
   3 tubes for blanks to determine background (B; only extracted with isooctane).

   Measurements are done in triplicates.

   If the sEH content is not known, prepare several dilutions of the tissue extract in the sodium phosphate buffer.

2. Add 100 μl tissue extract to each assay tube and 100 μl ice cold 0.1 M sodium phosphate buffer, pH 7.4/0.1 mg/ml BSA to each R and B tube.

   The BSA stabilizes pure sEH and helps retain lipophilic compounds in solution.

Run sEH assay

3. Begin timing the reaction ($t = 0$). Add 1 µl of 5 mM [^{3}H]*t*-SO solution in ethanol (50 µM final concentration) to the first set of triplicate tubes, using the Hamilton syringe equipped with a repeating dispenser. Mix 2 sec at high speed on a vortex and immediately place the tubes in a 30°C water bath.

4. Every 30 sec, repeat step 3 for each set of triplicate tubes.

   Starting and stopping the triplicate series every 30 sec ensures that every tube has been incubated for the same time.

5. At time *t* (generally 5 or 10 min), add 250 µl isooctane or hexanol (using a dispenser) to the first set of three replicates, mix thoroughly until an emulsion is formed (~10 sec), then put the tubes in the icy water.

   To increase sensitivity of the assay, longer incubation times could be used.

   The isooctane (Iso) is a nonpolar solvent that extracts only the hydrophobic epoxide substrate out of the water phase and leaves the diol and glutathione conjugates in the lower buffer hypophase. Because the isooctane is poorly miscible with the water, one must use extensive mixing of the phases to completely extract the epoxide.

   The hexanol (Hex) is more polar, and allows the extraction of both the epoxide and diol out of the water phase, leaving only the glutathione conjugates in the lower buffer phase.

   The difference between the two solvents gives the amount of diol produced. Thus, theoretically, one can monitor both glutathione-S-transferase and epoxide hydrolase activities in the same tube.

6. Every 30 sec, repeat step 5 for the additional triplicates, except for the total radioactivity (R) tubes, which are not extracted. Extract the blank (B) control only with isooctane.

7. Centrifuge the tubes 5 min at 1500× *g*, room temperature, to separate the phases.

8. Using a Hamilton syringe with a blunted needle, take a 30-µl sample of the aqueous (bottom) phase and transfer it to a 1.5-ml clear polypropylene microcentrifuge tube. Rinse the syringe with 95% ethanol between groups of replicates and wipe the tip to avoid cross-contamination.

   It is very important to use a glass syringe with a needle. Do not use a pipet with plastic tips because going through the organic phase that contains most of the radioactivity to reach the water phase will cause radioactive material to bind nonspecifically to the plastic tips and contaminate the samples. Further, it is very important to rinse the syringe with ethanol between series and to wipe the tip to avoid cross-contamination.

9. Add 1 ml scintillation liquid to the microcentrifuge tube, and mix with a vortex at high speed for a few seconds.

10. Measure the radioactivity (cpm) in each tube with a liquid scintillation counter.

Analyze data

11. Calculate the average for each triplicate. Calculate the sEH specific activity (nmol of diol formed/min/mg protein) as follows:

$$\frac{(\text{cpm}_{\text{UIso}} - \text{cpm}_{\text{Bk}}) \times 5\ \text{nmol} \times \text{dilution factor}}{0.92 \times \text{cpm}_{\text{R}} \times t \times 0.1\ \text{ml} \times \text{mg/ml protein}}$$

where cpm_{UIso} is the result obtained for the unknown tissue sample (U) after extraction with isooctane (Iso); cpm_{R} is the result obtained for the total radioactivity (R) tubes; 0.92 is a correction factor for the amount of diol (8%) that is extracted in the

isooctane phase; t is the incubation time in minutes; and cpm_{Bk} is the background correction (see below).

If cpm_B (obtained for the blank tubes) $\geq cpm_{UHex}$ (obtained for the unknown tissue sample after hexanol extraction), then $cpm_{Bk} = cpm_B$.

If $cpm_B \leq cpm_{UHex}$, then $cpm_{Bk} = cpm_{UHex}$.

The method is accurate if $3 \times cpm_B \leq cpm_{UIso} \leq 0.2 \times cpm_R$.

If $cpm_{UIso} < 3\times cpm_B$, the radioactivity measurement for the unknown sample is not statistically different from the background; a more concentrated enzyme solution and/or a longer incubation time are needed to obtain a higher value for cpm_{UIso}.

If $cpm_{UIso} > 0.2\times cpm_R$, too much of the substrate has been converted, and the amount of enzyme is not linearly proportional to the radioactivity measurement. Thus, the activity is being underestimated; a more diluted enzyme solution and/or a shorter incubation time should be used to obtain a lower cpm_{UIso}.

RADIOMETRIC ASSAY FOR sEH USING *trans*-DIPHENYL-PROPENE OXIDE (*t*-DPPO)

ALTERNATE PROTOCOL 1

The *trans*-diphenyl-propene oxide (*t*-DPPO) substrate is one that has been developed more recently (Borhan et al., 1995) to measure sEH activity. This method is two orders of magnitude more sensitive than the one described in Basic Protocol 1 in quantifying sEH activity. However, the substrate is not commercially available and must be synthesized (see Support Protocol 1).

Additional Materials (also see Basic Protocol 1)

5 mM [^{3}H]*trans*-diphenyl-propene oxide (*t*-DPPO) in dimethyl formamide, containing ~10,000 cpm/µl (see recipe)
HPLC-grade methanol

Perform the assay as in Basic Protocol 1 with changes to the following steps:

3. Add 1 µl of 5 mM [^{3}H]*t*-DPPO solution in ethanol (50 µM final concentration).

5. Add 60 µl of methanol and 250 µl isooctane or hexanol to the first set of tubes.

   The methanol is added to optimize the extraction efficiency of the diol in the aqueous phase (Borhan et al., 1995).

6. Extract the blank (B) control with 60 µl methanol and 250 µl isooctane only.

8. Take a 40-µl sample of the aqueous (bottom) phase and transfer it to a 1.5 ml microcentrifuge tube.

11. Use 0.91 as the correction factor for the amount of diol (9%) that is extracted in the isooctane phase.

SYNTHESIS OF *trans*-DIPHENYL-PROPENE OXIDE (*t*-DPPO)

SUPPORT PROTOCOL 1

The *t*-DPPO substrate is used for Alternate Protocol 1. The syntheses of both unlabeled and tritium-labeled compounds are similar (Borhan et al., 1995). Only the synthesis of the tritiated material is described; the unlabeled material could be obtained similarly by replacing the [$^3H_4$]$NaBH_4$ with $NaBH_4$ in step 5.

Materials

1,3-diphenyl-2-propanone
HPLC-grade carbon tetrachloride (CCl_4)
Sulfuryl chloride (SO_2Cl_2)
HPLC-grade ethanol
$NaBH_4$

0.1 M [$^3H_4$]$NaBH_4$ in ethanol (1.1 MBq/μl, 10.1 GBq/mmol)
1 M sodium hydroxide
HPLC-grade ethyl acetate
$MgSO_4$, anhydrous
Nitrogen gas tank
HPLC-grade acetonitrile

4-ml reaction vessels, dry (two)
Filter and filter paper (e.g., Whatman)
2-ml glass vial
Magnetic stir bar (small) and stirrer
65°C water bath
15-ml conical glass tube
HPLC equipped with 4 × 250–mm C18 reverse-phase column
Glass ampules

Prepare 1,3-diphenyl-1-choro-2-propanone

1. In a dry 4-ml reaction vessel, dissolve 1.05 g (5 mmol) 1,3-diphenyl-2-propanone in 2 ml CCl_4, add 0.85 g (6.3 mmol) SO_2Cl_2, and let the reaction proceed at room temperature overnight.
2. Filter the reaction mixture using filter paper to collect the precipitate (1,3-diphenyl-1-chloro-2-propanone).
3. Wash the white solid by pouring a few milliliters of CCl_4 through the filter on which the product is collected, and then dry the solid.

   A typical preparation yields 0.8 to 0.9 g of product.

Label the product

4. In a clean 2-ml glass vial dissolve 22 mg (0.09 mmol) 1,3-diphenyl-1-chloro-2-propanone in 50 μl ethanol and then add 60 μg $NaBH_4$.
5. In a clean and dry 4-ml reaction vessel, add 100 μl [$^3H_4$]$NaBH_4$ solution and then add the solution prepared in step 4.

   Unlabeled material can be synthesized by replacing the [$^3H_4$]$NaBH_4$ with $NaBH_4$ in this step.

6. Warm the reaction 3 min at 65°C, and then cool it to room temperature.
7. Mix 45 min at room temperature, using a small magnetic bar and stirrer.
8. Add 2 mg $NaBH_4$ dissolved in 200 μl of ethanol. Incubate 20 min at room temperature.
9. Slowly add 2 ml 1 M sodium hydroxide.
10. Allow the mixture to stand 1 hr at room temperature.

Extract the tritiated epoxide

11. Extract the tritiated epoxide with ethyl acetate as follows:

    a. Transfer the mixture from step 10 into a 15-ml conical glass tube.
    b. Add 2 ml ethyl acetate to the reaction vessel from step 10, and then transfer to the conical tube.
    c. Thoroughly mix until an emulsion is formed. Wait 5 min to let the phases separate.
    d. With a glass Pasteur pipet, transfer the ethyl acetate phase (top layer) to a clean tube.
    e. Repeat this step three times and pool together the three organic phases.

12. Dry the organic phase by adding 1 g anhydrous $MgSO_4$ to the ethyl acetate, and then filter the mixture into a clean tube. Wash the $MgSO_4$ on the filter with 2 ml ethyl acetate into the same tube.

13. Evaporate the organic solvent in the collection tube under a stream of nitrogen gas.

    CAUTION: *The solvent should be allowed to evaporate at slightly above room temperature, or much of the product can be lost.*

14. Dissolve the crude compound in 1 ml 35:65 acetonitrile/water.

Purify the labeled product

15. Inject the mixture on an HPLC column equipped with a reverse d-phase column and running an isocratic 35:65 acetonitrile/water solvent mixture.

    The [^{3}H]t-DPPO can be detected at 254 nm and elutes before its cis isomer.

16. Collect the [^{3}H]*t*-DPPO peak in a glass tube. Repeat until the entire crude compound solution (step 14) has been purified.

    This procedure will contaminate the HPLC injector and detector with tritium.

Extract the product

17. Extract the *t*-DPPO as follows:

    a. Measure the volume of the pooled eluted *t*-DPPO peak.
    b. Add 2 vol water and 2 ml ethyl acetate.
    c. Thoroughly mix until an emulsion is formed. Wait 5 min to let the phases separate.
    d. With a glass Pasteur pipet, transfer the ethyl acetate phase (top layer) to a clean tube.
    e. Repeat the extraction three times and pool together the three organic phases.

18. Collect the ethyl acetate and dry it as above, then transfer the liquid to a clean 15-ml conical glass tube.

19. Evaporate the organic solvent under a stream of nitrogen gas.

20. Add ethanol to obtain the desired concentration of [^{3}H]*t*-DPPO at ~250,000 cpm/µl. Store up to 1 year at −20°C.

    Longer term storage should be in hexane or pentane (rather than ethanol) with a trace of toluene. Furthermore, the solution needs to be sealed in a glass ampoule under a nitrogen or argon atmosphere. In such conditions, the compound is stable for 5 years at −80°C.

    For the unlabeled t-DPPO, prepare a 100 mM solution in N,N-dimethyl formamide (DMF). This solution is stable for over 5 years at −20°C. It is used to prepare the solution of tritiated t-DPPO (see recipe) used in Alternate Protocol 1.

MEASUREMENT OF sEH INHIBITION USING A FLUORIMETRIC ASSAY

BASIC PROTOCOL 2

This protocol (Jones et al., 2005) is a fast and accurate procedure for measuring sEH inhibition in vitro. This assay is based on an α-cyanocarbonate epoxide (CMNPC) that produces a strong fluorescent signal upon epoxide hydrolysis by sEH. While this assay is very sensitive for determining inhibition potency (IC_{50}), it requires the use of purified enzyme or at least a preparation free of competing enzyme activities (esterase and glutathione *S*-transferase). This assay is described using purified recombinant human sEH (see Beetham et al., 1993 and Wixtrom et al., 1988 for human sEH production and purification); however, it could be easily adapted to other EHs. (The protein concentration will need to be adjusted for other EHs.). Although, this assay describes a 96-well plate set-up, it is not totally appropriate for screening chemical libraries because of relative high

background caused by spontaneous hydrolysis of the substrate. A more stable substrate is needed for such screening (see Alternate Protocol 2).

Materials

Buffer A (see recipe), keep on ice
HPLC-grade DMSO
Inhibitors dissolved in DMSO at the appropriate concentration (e.g., 10 mM 12-(3-adamantan-1-yl-ureido)-dodecanoic acid; AUDA; see recipe)
1.2 μg/ml purified recombinant human sEH in Buffer A: prepare fresh and hold on ice
0.5 mM cyano(2-methoxynaphthalen-6-yl)methyl (3-phenyloxiran-2-yl)methyl carbonate (CMNPC; see recipe)
0.5 mM 6-methoxynaphthalene-2-carbaldehyde (MNC) in DMSO (for standard curve)
Black 96-well plates
Fluorimeter plate reader with temperature control, set at 30°C
50-μl glass Hamilton syringe equipped with a repeating dispenser

Prepare 96-well plates

1. In a black 96-well plate, fill all the wells with 150 μl buffer A.
2. Using the repeating Hamilton syringe, add 2 μl DMSO in the control blank (B) and control total activity (T) wells. In the test wells, add 2 μl inhibitor solution in DMSO.

   Because of well-to-well variation, measurements at least in triplicate wells are recommended.

3. Mix the plate, and warm it to 30°C in the plate reader.
4. Add 20 μl buffer A in the blank control (B) wells, and add 20 μl 1.2 μg/ml purified recombinant human sEH in buffer A in the total activity (T) and test wells containing inhibitor (I).
5. In the plate reader, mix the plate 10 sec and incubate 5 min at 30°C.
6. During incubation, prepare the working substrate solution of substrate by mixing 3.464 ml buffer A with 266 μl of 0.5 mM CMNPC.

Run assay

7. Add 30 μl working substrate solution to all the wells (5 μM final concentration).
8. Put the plate in the reader, mix at least 10 sec, and start the fluorescence reading.
9. Read with an excitation wavelength of 330 nm, an emission wavelength of 465 nm, and a cut-off at 450 nm every 30 second for 10 min.

Analyze data

10. Use the velocities in milli relative fluorescence units (mRFU)/min to calculate the percentage of inhibition as follows:

$$100 \times \left[1 - \frac{(V_I - V_B)}{(V_T - V_B)} \right]$$

where V_I is the velocity obtained in the presence of an inhibitor (I); V_B is the velocity obtained for the blank (B) control; and V_T is the velocity obtained for the total activity (T) control (no inhibitor).

The linearity of the reaction rate should be checked.

11. Determine the concentration of inhibitor that gives 50% inhibition (IC_{50}) by plotting the percent inhibition as a function of the inhibitor concentration.

Determine activity

12. Prepare a standard curve as follows:

    a. Prepare seven dilutions (final concentrations between 0.005 and 0.1 mM) of the 0.5 mM solution of 6-methoxynaphthalene-2-carbaldehyde (MNC, the reaction product) in DMSO.
    b. In a black 96-well plate, fill 24 wells (eight triplicates) with 198 µl buffer A. Add 2 µl DMSO in the first triplicate set up wells, and then 2 µl of each dilution of MNC in the following wells in triplicate.
    c. Measure the fluorescence with an excitation wavelength of 330 nm, an emission wavelength of 465 nm, and a cut-off at 450 nm.
    d. Plot the fluorescence readings as a function of the MNC concentration.

13. Use the standard curve from step 12 to convert the fluorescence readings (mRFU/min) to enzyme activity measurements (nmol MNC formed/min).

FLUORIMETRIC ASSAY FOR THE SCREENING OF CHEMICAL LIBRARIES

ALTERNATE PROTOCOL 2

An α-cyanoester epoxide (PHOME), which displays high aqueous stability and solubility, is used for a high-throughput screening (HTS) assay with long incubation times at room temperature (Wolf et al., 2006). This assay is less sensitive in terms of distinguishing potent inhibitors than the one described in Basic Protocol 2. This assay is described using purified recombinant human sEH; however, it could be easily adapted to other EHs, e.g., with adjustments to the protein concentration. The substrate used for the assay is not commercially available and must be synthesized (see Support Protocol 2).

Additional Materials *(also see Basic Protocol 2)*

Inhibitors dissolved in DMSO at the appropriate concentration [e.g., 10 mM 12-(3-adamantan-1-yl-ureido)-dodecanoic acid; AUDA; see recipe]
0.4 µg/ml purified recombinant human sEH in buffer A
5 mM (3-phenyl-oxiranyl)-acetic acid cyano-(6-methoxy-naphthalen-2-yl)-methyl ester (PHOME; see recipe)
1 M $ZnSO_4$ in buffer A (see recipe): prepared just before use

Set up plates

1. Dilute the inhibitor solutions in buffer A to a concentration of 10-fold the targeted concentration to be tested

   Test solutions should not contain more than 10% DMSO.

   For example, to test a final concentration of 100 nM, prepare 1 µM solutions.

2. In a black 96 well plate, dispense 20 µl inhibitor solutions in the test wells.
3. In the blank (B) control wells and total activity (T) control wells dispense 20 µl buffer A.

   The authors suggest doing at least triplicate measurements each for the blank and total activity controls.

4. Add 2 µl of inhibitor solution (e.g., 10 mM AUDA) in the blank (B) control wells.

Run assay

5. Add 130 µl of 0.4 µg/ml purified recombinant human sEH enzyme solution to all the wells.
6. Mix the plate, and incubate 5 min at room temperature.
7. During incubation prepare the working solution of substrate by mixing 0.4 ml of the 5 mM PHOME stock solution with 9.6 ml of buffer A (final concentration 200 µM).
8. Add 50 µl working substrate solution to all the wells (5 µM final concentration).
9. Mix 1 min, and then incubate 90 min at room temperature (22°C to 25°C).

   A good signal-to-noise ratio is obtained with between 60 and 120 min of incubation.

10. Add 50 µl of 1 M $ZnSO_4$ to all the wells to stop the reaction.
11. Read fluorescence with an excitation wavelength of 315 nm, an emission wavelength of 460 nm, and a cut-off at 450 nm.

Analyze data

12. Use the fluorescent measurements (mRFU) to calculate the percentage inhibition as follows:

$$100 \times \left[1 - \frac{(F_I - F_B)}{(F_T - F_B)}\right]$$

where F_I is the fluorescence signal obtained in the presence of an inhibitor (I); F_B is the fluorescence signal obtained for the blank (B) control; and F_T is the fluorescence signal obtained for the total activity (T) control (no inhibitor).

13. Ensure the linearity of the activity over the time of the experiment. Calculate an IC_{50} and transform the fluorescence readings to enzyme activity as in Basic Protocol 2, steps 11 to 13.

SUPPORT PROTOCOL 2

SYNTHESIS OF (3-PHENYL-OXIRANYL)-ACETIC ACID CYANO-(6-METHOXY-NAPHTHALEN-2-YL)-METHYL ESTER (PHOME)

The PHOME substrate is used for Alternate Protocol 2. The synthesis of this compound is described by Jones et al. (2005).

Materials

trans-styrylacetic acid
Hydroxyl-(6-methoxy-naphthalen-2-yl)-acetonitrile
N-ethyl-N′-dimethylaminopropyl-carbodiimide (EDCI)
N,N-dimethyl-4-amino-pyridine (DMAP)
HPLC-grade dichloromethane
1 M potassium carbonate
$MgSO_4$, anhydrous
HPLC-grade ethyl acetate, toluene, and hexane
meta-chloro-perbenzoic acid (m-CPBA)

50-ml reaction vessels, dry (two)
50-ml separatory funnel
50-ml Erlenmeyer flask
50-ml round-bottom flask
1 × 20–cm silica gel column (for preparation see Vogel, 1989)
Vacuum source

Carry out reaction

1. In a dry reaction vessel, weigh 460 mg (1.75 mmol) of *trans*-styrylacetic acid, 355 mg (1.65 mmol) of hydroxyl-(6-methoxy-naphthalen-2-yl)-acetonitrile, 330 mg (1.75 mmol) EDCI, and 42 mg of DMAP (0.34 mmol).
2. Add 15 ml dichloromethane, and stir 36 hr at room temperature.
3. Wash the reaction mixture twice with 20 ml of 1 M potassium carbonate solution as follows:
   a. Transfer the mixture from step 2 into a 50-ml separatory funnel.
   b. Add 20 ml of a 1 M potassium carbonate solution.
   c. Thoroughly mix the phases in the funnel until an emulsion is formed. Wait 5 min to let the phases separate.
   d. Collect the organic phase (bottom) into a clean Erlenmeyer flask and discard the aqueous phase.
   e. Repeat this step one more time.
4. Dry the organic (dichloromethane) phase by adding 5 g anhydrous $MgSO_4$ and filtering into a clean round-bottom flask. Wash the $MgSO_4$ on the filter with 10 ml dichloromethane into the same flask. Evaporate the solvent under vacuum.

Column purify the ester

5. Prepare a 1 × 20–cm silica gel column and equilibrate with 100 ml pure hexane.
6. Dissolve the dried material from step 4 in 10 ml hexane and chromatograph on the silica column, eluting with a 3:1 toluene/hexanes solvent mixture.
7. Evaporate the solvent under vacuum to obtain cyano-(6-methoxy-naphthalen-2-yl)methyl *trans*-{[2-(2-phenylethenyl)]} acetate as a yellowish oil.

   Generally, this method yields ~700 mg of ester.

Treat ester with m-CPBA

8. In a dry clean vessel, dissolve the ester oil from step 7 in 10 ml of dichloromethane.
9. While stirring, slowly add 516 mg (3.0 mmol) *m*-CPBA.
10. Stir the reaction 3 days at room temperature, in the dark.
11. Wash the reaction mixture three times with 10 ml 1 M potassium carbonate solution as in step 3.
12. Dry the organic phase as in step 4 and then evaporate the solvent under vacuum.

Isolate PHOME

13. Prepare a 1 × 20–cm silica gel column and equilibrate with 100% hexane.
14. Chromatograph the residue from step 12 on the silica column eluting with a 15:85 ethyl acetate/hexanes solvent mixture.
15. Evaporate the solvent to obtain the PHOME compound as a white solid.

    Generally, this method yields ~100 mg of PHOME.

    Be sure to check the background fluorescence of the product and chromatographically monitor for the presence of the 6-methoxynaphthalene-2-carbaldehyde impurity.

BASIC PROTOCOL 3

LC-MS/MS METHODS FOR MEASURING sEH ACTIVITY

Because numerous natural epoxides are neither absorbent nor fluorescent and are not available in a radioactive form, it is very difficult to measure the activity of sEH for these substrates. A LC-MS/MS method was developed (Newman et al., 2002) to quantify epoxides and diols of unsaturated fatty acids. While the assay described in this protocol is for 14,15-epoxyeicosatrienoic acid (14,15-EET), this method can be adapted to any epoxide-containing compound as long as there is a method for detecting and quantifying the diol formed. Further, the assay described in this protocol uses purified human sEH, but it can be modified for use with other EHs and tissue extracts (e.g., by adjusting the protein concentration).

Materials

Buffer A (see recipe), ice cold
0.50 mM 14,15-dihydroxy-eicosatrienoic acid (14,15-DHET; Cayman Chemicals; see recipe), ice cold
HPLC-grade methanol, ice cold
0.25 mM 9,10-dihydroxy-octadec-12-enoic acid (9,10-DiHOME; Cayman Chemicals) in ethanol (see recipe; internal standard), ice cold
0.2 μg/ml recombinant purified human sEH at in buffer A, ice cold
5 mM 14,15-epoxyeicosatrienoic acid (14,15-EET; Cayman Chemicals) solution in ethanol (see recipe)
Standard solutions of 14,15-dihydroxy-eicosatrienoic acid (14,15-DHET; Cayman Chemicals; see recipe)
10 × 75–mm borosilicate glass tubes
30°C water bath
Timer
50-μl glass Hamilton syringe equipped with a repeating dispenser
Sampling vials for LC-MS/MS
HPLC equipped with a tandem quadrupole mass spectrometry detector (MS/MS)

Prepare a standard curve

1. To each of seven 10 × 75–mm borosilicate tubes, add 100 μl buffer A.
2. Using the repeating Hamilton syringe, add 1 μl of 14,15-DHET to each individual tube, and mix on a vortexer for a few seconds.
3. Add 400 μl of methanol to each tube and 1 μl of 25 mM 9,10-DiHOME (internal standard), then vortex at high speed for a few seconds.
4. Transfer the contents of each tube to sampling vials for LC-MS/MS.
5. Analyze the 9,10-DiHOME and 14,15-DHET by LC-MS/MS. For the 9,10-DiHOME use the 313 > 201 parent to daughter ion transition, and for the 14,15-DHET use the 337 > 207 parent to daughter ion transition.
6. Determine the standard curve by drawing the ratio $A_{14,15\text{-DHET}}/A_{9,10\text{-DiHOME}}$ as a function of the concentration of 14,15-DHET (final concentrations 0, 0.1, 0.2, 0.3, 0.5, 0.7 and 1.0 μM). Where $A_{14,15\text{-DHET}}$ is the area for 14,15-DHET peak, and $A_{9,10\text{-DiHOME}}$ is the area for 9,10-DiHOME peak.

   A linear curve should be obtained.

Carry out sEH reaction

7. Place in icy water as many 10 × 75–mm glass tubes as required to perform three replicates for each test condition.
8. Add 100 μl of 0.2 μg/ml recombinant purified human sEH to each tube.

If the sEH content is not known prepare several dilutions of the enzyme preparation in buffer A.

9. Begin timing ($t = 0$) the assay. Immediately add 1 µl of 5 mM 14,15-EET solution in ethanol (50 µM final concentration), to the first set of triplicate tubes, using the Hamilton syringe equipped with a repeating dispenser. Mix 2 sec at high speed on a vortexer and immediately place the tubes in a 30°C water bath.
10. Every 30 sec, repeat step 9 for each additional set of triplicate tubes.

    By starting and stopping the triplicate series every 30 sec, you ensure that every tube has been incubated the same time.

11. At time *t* (generally 5 or 10 min), add 400 µl methanol to each tube, and 1 µl of the 9,10-DiHOME (internal standard), vortex for few seconds, and put the tubes in icy water.
12. Every 30 sec, repeat step 11 for the following triplicates.

Analyze the results

13. Transfer the content of each tube to sampling vials for LC-MS/MS.
14. Analyze the 9,10-DiHOME and 14,15-DHET by LC-MS/MS using the following transitions: for the 9,10-DiHOME use the 313 > 201 parent to daughter ion transition, and for the 14,15-DHET use the 337 > 207 parent to daughter ion transition.
15. For each sample, determine the µM 14,15-DHET, using the ratio $A_{14,15\text{-DHET}}/A_{9,10\text{-DiHOME}}$ from the standard curve, using prepared standard solutions.
16. Calculate the sEH specific activity (nmol of diol formed/min/mg protein) as follows.

$$\frac{\mu\text{M 14,15-DHET} \times 0.5\text{ ml}}{t \times 0.1\text{ ml} \times \text{mg/ml protein}}$$

This method is accurate if <10% of the substrate has been consumed.

REAGENTS AND SOLUTIONS

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

12-(3-adamantan-1-yl-ureido)-dodecanoic acid, 10 mM

In a small glass vial, dissolve 3.7 mg of 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA; Cayman Chemicals) in 1 ml of DMSO. Store up to 6 months at −20°C.

Do not use a plastic tube, because AUDA may be absorbed to plastic. Upon melting from storage, it is necessary to resolubilize AUDA by vigorously mixing the solution.

Buffer A

Dissolve 5.23 g of 2{bis[2-hydroxyethyl(imino)-2-(hydroxymethyl]}-1,3-propanediol (bis/Tris) in 400 ml of water. Adjust the pH to 7.0 at room temperature with 1 M HCl. Adjust with water to a final volume of 1 liter. Store up to 1 year at room temperature.

Just before use, dissolve 5 mg fraction V bovine serum albumin (Sigma) in 50 ml of the buffer.

This buffer is 25 mM bis-Tris·HCl, pH 7.0, containing 0.1 mg/ml of BSA.

Cyano(2-methoxynaphthalen-6-yl)methyl (3-phenyloxiran-2-yl)methyl carbonate, 0.5 mM

In a 20-ml glass vial, weigh 3.9 mg of cyano(2-methoxynaphthalen-6-yl)methyl (3-phenyloxiran-2-yl)methyl carbonate (CMNPC or Epoxy Fluor 7; Cayman Chemicals). Add 20 ml of DMSO. Prepare just before use.

Do not use a plastic tube, because CMNPC may be absorbed to plastic.

Make sure that the preparation has a low fluorescent background before starting measurements.

9,10-dihydroxy-octadec-12-enoic acid, 0.25 mM

In a small glass vial, dissolve 25 μg of 9,10-dihydroxy-octadec-12-enoic acid (9,10-DiHOME; Cayman chemicals) in 320 μl of 100% ethanol. Store up to 6 months at −20°C, under an inert gas.

9,10-DiHOME is sensitive to air, be sure to keep it under an inert gas.

14,15-epoxyeicosatrienoic acid, 5 mM

In a small glass vial, dissolve 500 μg of 14,15-epoxyeicosatrienoic acid (14,15-EET; Cayman Chemicals) in 310 μl of 100% ethanol. Store up to 6 months at −20°C, protected from air under an inert gas.

14,15-EET is sensitive to air, be sure to keep it under an inert gas.

[^{3}H]trans-diphenyl-propene oxide solution, 5 mM, ∼10,000 cpm/μl

Prepare a 100 mM solution of cold *trans*-diphenyl-propene oxide (*t*-DPPO) in *N,N*-dimethyl-formamide (DMF). Mix 100 μl of the cold *t*-DPPO solution with 1.820 ml DMF, and 80 μl [^{3}H]*t*-DPPO at ∼250,000 cpm/μl in ethanol (see Support Protocol 1). Store up to several years at −20°C.

Use a freshly opened bottle of DMF; oxidation products accumulate with time in opened DMF bottles, and they may inhibit the sEH activity. Because of the tritium half- life (∼10 years), this substrate solution needs to be prepared anew every 5 years for best results.

[^{3}H]trans-stilbene oxide solution at 5 mM, ∼10,000 cpm/μl

Prepare a 100 mM solution of cold *trans*-stilbene oxide (*t*-SO); Aldrich) in 100% ethanol. Mix 200 μl of the cold *t*-SO solution with 3.780 ml 100% ethanol and 20 μl [^{3}H]*t*-SO at 1 mCi/ml in ethanol (American Radiolabeled Chemicals). Store up to several years at −20°C.

Do not use 95% ethanol; such solvent is acidic and will hydrolyze the epoxide. Because of the tritium half-life (∼10 years), this substrate solution needs to be prepared anew every 5 years for best results.

(3-phenyl-oxiranyl)-acetic acid cyano-(6-methoxy-naphthalen-2-yl)-methyl ester, 5 mM

In a 20 ml glass vial, weigh 38.7 mg of (3-phenyl-oxiranyl)-acetic acid cyano-(6-methoxy-naphthalen-2-yl)-methyl ester (PHOME; see Support Protocol 2). Dissolve it in 20 ml of DMSO. Prepare just before use.

Do not use a plastic tube, because PHOME may be absorbed to plastic.

Standard solutions of 14,15-dihydroxy-eicosatrienoic acid

In a small glass vial, dissolve 100 μg of 14,15-dihydroxy-eicosatrienoic acid (14,15-DHET; Cayman Chemicals) in 590 μl of 100% ethanol to form the 0.50 mM solution of 14,15-DHET. Store up to several years at −20°C under an inert gas.

14,15-DHET is sensitive to air.

In five glass vials, dispense 90, 80, 70, 50 and 30 μl ethanol, and then add 10, 20, 30, 50, and 70 μl of 0.50 mM 14,15-DHET to form the 0.05, 0.10, 0.15, 0.25, and 0.35 mM solutions of 14,15-DHET, respectively.

COMMENTARY

Background Information

Epoxide-containing compounds are ubiquitously found in the environment from both natural and man-made sources, and a large variety of aromatic and alkenic compounds are also metabolized to epoxides endogenously (Oliw, 1994). The catalytic addition of water to epoxides or arene oxides by epoxide hydrolases (EH, E.C.3.3.2.3) to yield the corresponding 1,2-diols or glycols (Oesch, 1973) is only one of several ways that cells transform oxiranes. In mammals, there are several epoxide hydrolases—including the soluble epoxide hydrolase (sEH) and microsomal epoxide hydrolase (mEH)—that have been the most studied EHs over the past 30 years (for a recent review see Newman et al., 2005). These two enzymes are described as complementing each other in detoxifying a wide array of xenobiotic epoxides (Hammock et al., 1997); however recent findings clearly implicate the sEH in the regulation of blood pressure, pain, and inflammation through the hydrolysis of endogenous epoxy fatty acid such as 14,15-EET. See Imig (2006) and Spector and Norris (2007) for recent reviews on the physiological role of sEH. Expression of sEH is well known to be increased by peroxisome proliferator-activated receptor (PPAR)-alpha agonists, such as fibrates commonly used as hypolipidemic drugs or phthalates commonly used as plasticizers (Grant et al., 1994). Further, the sEH activity is inhibited by urea-containing compounds, such as the herbicide Siduron or the topical antiseptic trichlorocarban (Morisseau et al., 1999; McElroy et al., 2003) as well as some benzoylurea insecticides (Nakagawa et al., 2000). Taken together, these findings underline how environmental contaminants could influence human health through induction or inhibition of sEH.

Historically, sEH was first reported to hydrolyze tri-substituted terpenoid epoxides called juvenile hormones, which affect insect development (Hammock et al., 1976). Based on the large polarity difference between the epoxide and diol, a rapid partition assay, similar to the one described in Basic Protocol 1, was developed for [^{3}H]-labeled juvenile hormone III (JH-III; Mumby and Hammock, 1979). However, because of relatively low turnover, this method is not desirable for the sensitive detection of sEH activity (limit of detection is ∼10 μg/ml for purified human sEH; Morisseau et al., 2000). In an effort to obtain a more sensitive assay, JH-III (or its analogs) was replaced by [^{3}H]-labeled *trans*-β-ethylstyrene oxide (Mullin and Hammock, 1980) or [^{3}H]-labeled *trans*-stilbene oxide (Gill et al., 1983). In both cases, the large polarity difference between the epoxide and diol allowed a rapid partition assay (the assay for [^{3}H]-labeled *trans*-stilbene oxide is described in Basic Protocol 1). While these two assays are more sensitive (limits of detection are ∼1 and 5 μg/ml of purified human sEH, respectively); two orders of magnitude increase in sensitivity (*t*-DPPO limit of detection of ∼50 ng/ml of purified human sEH) was obtained using [^{3}H]-labeled *trans*-diphenylpropene oxide (Borham et al., 1995; see Alternate Protocol 1).

While these radioactive assays are very sensitive for the measurement of sEH activity, they are quite expensive. Thus, early on, a continuous spectrophotometric assay was developed using *trans*-stilbene oxide as substrate (Hasegawa and Hammock, 1982). Because this assay is based on the difference in the ultraviolet absorbance of the substrate and diol product, it is not very sensitive. While this method was improved using aromatic epoxides that sEH turns over at rates 5- to 100-fold higher than *t*-SO (Wixtrom and Hammock, 1988), a faster and more sensitive spectrophotometric assay was obtained with the use of 4-nitrophenyl-2,3-epoxy-3-phenylpropyl carbonate (NEPC; Dietze et al., 1994). Upon hydrolysis of the NEPC epoxide, the diol formed spontaneously cyclizes to

free 4-nitrophenol, which is yellow and could be followed at 405 nm, with its appearance being proportional to diol formation. While this assay is sensitive (NEPC limit of detection is ~1 μg/ml of purified human sEH), it requires the use of purified enzyme because NEPC is also a substrate for glutathione *S*-transferases and carboxylesterases (Dietze et al., 1994). This method was improved by replacing the 4-nitrophenol with hydroxyl-(6-methoxy-naphthalen-2-yl)-acetonitrile, which rapidly decomposes to the highly fluorescent 6-methoxy-2-naphthaldehyde (Jones et al., 2005). This assay is very sensitive for sEH (CMNPC limit of detection is ~10 ng/ml of purified human sEH), but like the NEPC assay, it works best with the purified enzyme. This assay is mainly used to determine the potency of inhibitors (see Basic Protocol 2).

The measurement of sEH activity with natural epoxides, especially epoxy fatty acids, is complicated because the epoxides or diols are neither absorbent nor fluorescent, and the polarity difference between the epoxides and diol is small, making it impossible to develop a partition assay such as the one described in Basic Protocol 1. Thus, to use [^{14}C]-labeled epoxy-fatty acids, one needs to extract both the epoxide and diol formed at the end of the enzymatic reaction and then separate them by thin layer chromatography before measuring the radioactivity of the formed diol (Gill and Hammock, 1979; Zeldin et al., 1993; Greene et al., 2000). These methods are obviously time-consuming. The development of an HPLC-MS/MS method to simultaneously detect both the epoxides and diols of fatty acids (Newman et al., 2002) was key to obtaining a faster assay for sEH natural substrates (see Basic Protocol 3).

Critical Parameters

For the radioactive assays in Basic Protocol 1, Alternate Protocol 1, and Basic Protocol 3, time and temperature are critical parameters. Because sEH is still active at 0°C (Morisseau and Hammock, 2005), it is very important to make sure that every tube is incubated the same length of time with the enzyme. Further, the time of incubation is the easiest parameter to change to ensure that substrate hydrolysis is in the optimal linear range.

For Basic Protocol 2 and Alternate Protocol 2, the purity of the enzyme preparation and the pH of the buffer are very important parameters. Because these fluorescent substrates could undergo turnover by glutathione *S*-transferase and carboxylesterase, it is important to use a preparation free of these enzymes. The substrates used (CMNPC or PHOME) contain an epoxide that is sensitive to acidic pH, and they contain a carbonate or ester that is sensitive to basic pH. Thus, the reaction should be done with these substrates using a pH between 7.0 and 8.0.

Troubleshooting

For Basic Protocol 1 and Alternate Protocol 1, the most common problem is that the substrate hydrolysis rate is outside the optimal linear range ($cpm_U > 0.2 \times cpm_R$). Too much of the substrate has been converted, and the sEH activity is underestimated. One needs to use a more diluted enzyme solution and/or use a shorter incubation time. When using very concentrated cellular extracts (>10 mg/ml protein), a thick interface appears, making it difficult to take a sample of the aqueous phase (step 8). To avoid any problem, slowly lower the blunted needle of the syringe down the wall of the glass tube, and then gently push away the interfacial mixture. Finally, lower the syringe needle down to the bottom of the tube, then aspirate and expel some of the aqueous phase before taking the sample for radioactivity counting. Taking slightly more water phase sample than is required, expelling the extra sample after the needle is out of the liquid, and cleaning the tip of the needle with a wipe will help reduce further contamination and background.

The most common problem for Basic Protocol 2 and Alternate Protocol 2 is a high fluorescent background. This is due to a poor purification of the substrate or to hydrolysis upon storage. Be sure to prepare the CMNPC and PHOME solutions just before use. Another classic problem for these protocols is the binding of the fluorescent substrates to plastic or to residual mold-releasing agents used in manufacturing plastics. Be sure to prepare these substrates in glass containers. Adding the substrate solution in DMSO to buffer containing some BSA will reduce absorbance to plastic.

The sEH activity is very sensitive to the presence of detergent. Detergent will inhibit the activity by denaturing the enzyme and trapping the substrate in micelles. Because sEH only hydrolyzes soluble substrates (Wixtrom and Hammock, 1985), the formation of micelles will result in smaller amounts of soluble substrate. Avoid using detergent for the preparation of cell extracts destined for sEH activity measurement. Otherwise, dilute the enzyme preparation to ensure that the concentration of

detergent is less than 0.01% or below the critical micelle concentration.

Anticipated Results

The choice of either radioactive *t*-SO or *t*-DPPO to measure sEH activity is mainly dependent on the tissue extract sEH content. The *t*-SO assay (Basic Protocol 1) will be accurate for concentrations of sEH above a few micrograms per milliliter; for lower concentrations the *t*-DPPO assay (Alternate Protocol 1), with a limit of detection of ~50 ng/ml for human sEH, should be used. Alternatively, one could use an LC-MS/MS assay (Basic Protocol 3), which also has a limit of detection of ~50 ng/ml for purified human sEH. However, this method requires access to a tandem quadrupole mass spectrometer, which is not yet a common laboratory instrument. However, this assay could be adapted to simple quadrupole instruments or GLC-MS.

The fluorescence assays (Basic Protocol 2 and Alternate Protocol 2) are designed to measure inhibition potency (IC_{50}) of chemicals against purified sEH. These methods are accurate (SD < 20%) for IC_{50} between 1 and 100,000 nM.

Time Considerations

For all assays, the total time required depends on the duration of the reaction and the number of samples. In general, for all assays except for Alternate Protocol 2, the enzymatic reaction is complete in <15 min. The PHOME assay takes ~1 hour. The radioactive assays take a couple of minutes to measure the radioactivity of one sample. For the LC-MS/MS method, it takes ~5 min to determine the diol concentration of one sample.

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Key References

Borhan et al., 1995. See above.

This method is described in Alternate Protocol 1.

Jones et al., 2005. See above.

This method is described in Basic Protocol 2.