S-MEPHENYTOIN 4-HYDROXYLATION ASSAY

This assay measures the P450-mediated metabolism of S-mephenytoin (S-MEPH), an anticonvulsant agent, to 4-hydroxymephenytoin (4-OH MEPH). In human liver, this reaction is catalyzed **exclusively by CYP2C19.** S-MEPH is also converted to a second metabolite, nirvanol, by human liver microsomes but this reaction is mediated by CYP2C9 (see below).

I. REAGENTS NEEDED

A) 100 mM potassium phosphate buffer, **pH 7.4**, at room temperature.

B) 9 mM S-MEPH Prepare by dissolving 5 mg solid S-MEPH solid (Cayman 11913) in 2.5 ml MeOH. Store @ -20°C.

C) **Buffer/Substrate** Prepare by adding 75 ul 9 mM S-MEPH (Reagent B) to 4.925 ml buffer (Reagent A). S-MEPH concentration = 120 µM; enough for 20 assay tubes.

D) 10 mM NADPH Prepare by dissolving 4.7 mg in 0.5 ml Buffer A – enough for 20 assay tubes.

E) Ethyl Acetate

F) 2 N HCl

II. PROCEDURE

1. Pipette appropriate amount of **Reagent C** containing 120 µM S-MEPH into 1.5 ml Eppendorf-type microfuge tubes, keeping in mind that the final assay volume is **0.25 ml**. Place tubes in an ice bath.

2. Add either microsomes (**0.1** nmol microsomal P450) or purified reconstituted P450 (20-50 pmol) to the tubes.

3. Add 25 µl of **Reagent D** to the appropriate tubes, vortex, and place tubes in shaking water bath at 37°C.

DO NOT ADD Reagent D to incubation tubes that will be used as blanks or for standards.

4. Terminate the reactions after **45 min** by adding 1.0 ml of **Reagent E** to the tubes and briefly vortexing.

5. Once all of the reactions are terminated, add 100 µl of **Reagent F** to each tube and vortex for 5 min using the VWR Multiple Vortexer.

6. Centrifuge tubes at 10,000 rpm for 5 min in the Heraeus Microfuge to separate the organic and aqueous layers. Transfer 0.70 ml of the organic (upper) phase to a 10 x 75 mm disposable glass tubes, and evaporate sample to dryness at room temp using the N2-EVAP nitrogen evaporator.

7. Add 60 µl of ACN:H_3PO_4 (1:1, v/v) to each tube, cap the tubes, vortex vigorously, and sonicate for 5 min to **COMPLETELY RESOLUBILIZE** the residues. Subject samples to HPLC analysis or store @ -20°C (see below).

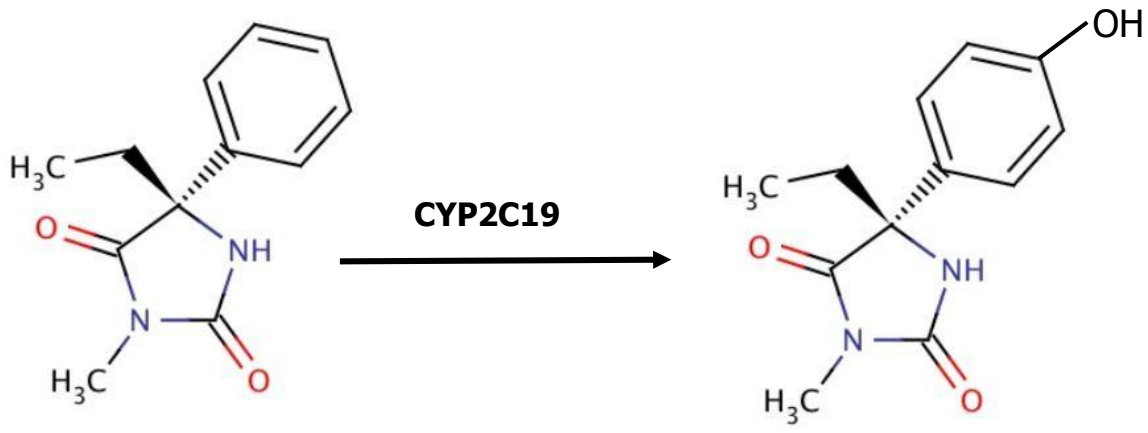
III. HPLC ANALYSIS

Column:	Phenomenex Kinetex column (4.6 x 150 mm) w/CrudCatcher Guard Column
Mobile Phase:	**Solvent A** – 0.05% H_3PO_4 (phosphoric acid✣), pH 2.6
	Solvent B – 100% Acetonitrile
Flow Rate:	0.5 ml/min
Column Temp:	Ambient
Sample Temp:	Ambient
Peak Detection	212 nm
Injection Volume	**30 µl**
Run Time	18 min

Gradient:

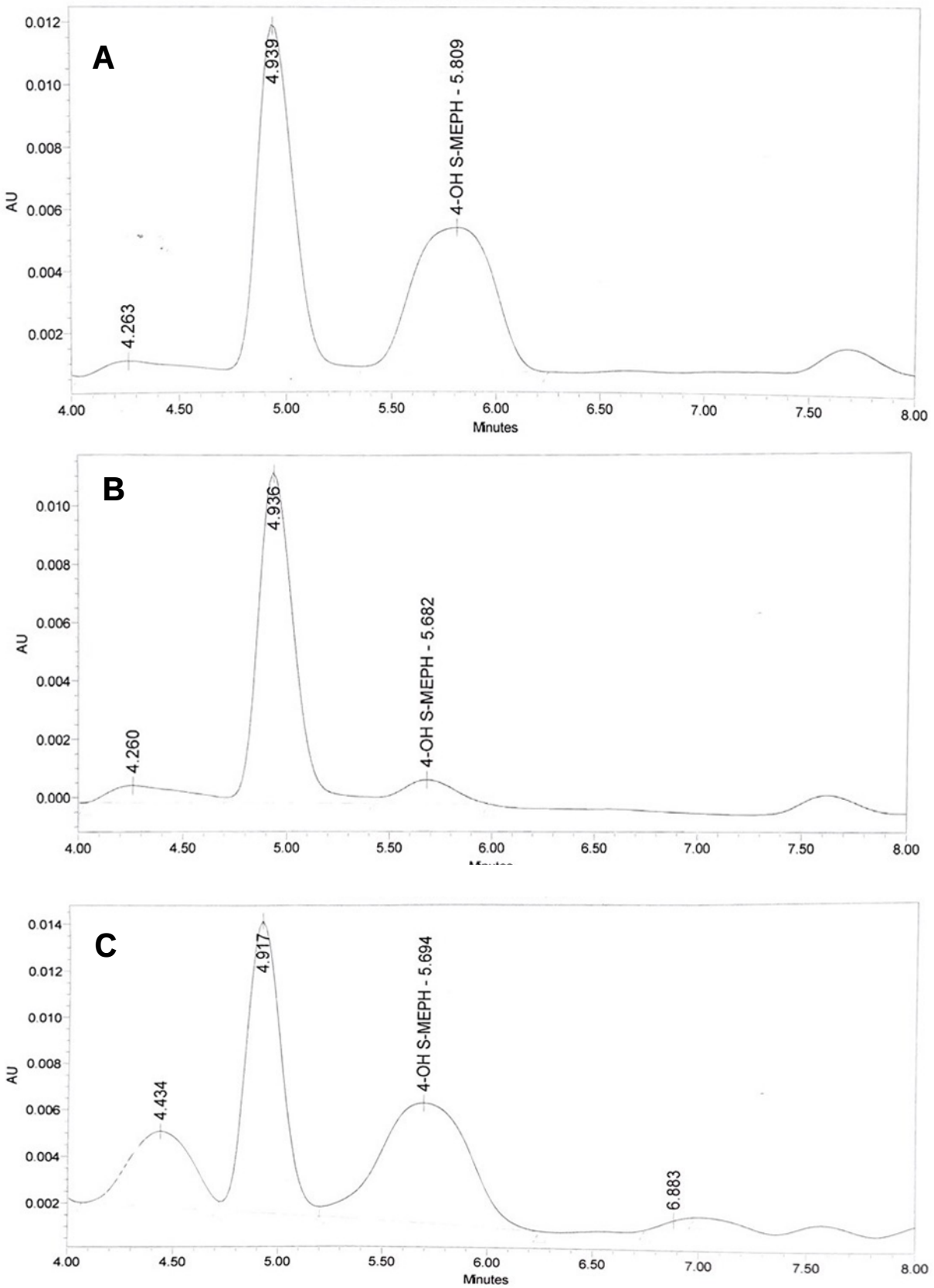
Time (min)	Solvent A (%)	Solvent B (%)	Flow Rate (ml/min)	Curve
Initial	80	20	0.75	
6.0	80	20	0.75	11
11.0	25	75	0.75	2
13.1	80	20	0.75	1

Under these HPLC conditions, 4-OH MEPH and S-MEPH elute at 5.9 and 9.9 min, respectively.



S-MEPHENYTOIN **4-HYDROXYMEPHENYTOIN**

S-MEPHENYTOIN - CYP2C19



HPLC analysis of *S*-mephenytoin 4'-hydroxylation by human liver microsomes. S-mephenytoin (*S*-MEPH, 120 µM) was incubated with human liver microsomes (0.24 mg protein, 0.1 nmol aggregate P450) for 45 min at 37°C in the presence of NADPH plus 0.5 mg preimmune IgG, 0.5 mg anti-CYP2C19 IgG or 0.05 mg anti-CYP2C9 mAb. *S*-MEPH was then resolved from the 4-hydroxymephenytoin (4-OH-MEPH) metabolite by reversed-phase HPLC as described above using a Waters Alliance unit equipped with a model 2690 separation module and a model 2487 UV/VIS detector.
A Phenomenex Kinetex C18 column (4.6 mm x 15 cm, 5 µm particle size) was utilized. Gradient elution with a mobile phase consisting of acetonitrile:0.05% phosphoric acid was performed employing a flow rate of 0.75 ml/min, and the column eluates were continuously monited for ultraviolet absorbance at 212 nm. Under these conditions, 4-OH S-MEPH and S-MEPH exhibited retention times of 5.9 and 9.9 min, respectively with a total sample analysis time of 18 min; the *S*-MEPH peak is not shown here. 4-OH S-MEPH formation was quantified by comparison of peak areas with those of analytical standard curve.

a) *S*-MEPH + liver microsomes + NADPH + Preimmune IgG
b) *S*-MEPH + liver microsomes + NADPH + Anti-CYP2C19 IgG
c) *S*-MEPH + liver microsomes + NADPH + Anti-CYP2C9 mAb