NIFEDIPINE OXIDATION ASSAY

This method measures the P450-dependent oxidation of nifedipine, a calcium channel blocking agent, to its pyridine metabolite. Nifedipine oxidation in human liver microsomes is catalyzed by CYP3A4 (85%) and CYP3A5 (15%), both of which are inducible by agents that are PXR ligands (e.g., rifampicin)

⌧**IMPORTANT NOTE**: Solutions containing nifedipine are **light sensitive**. Therefore, perform assay under reduced lighting if possible – do not expose tubes to bright, direct light.

I. REAGENTS NEEDED

A) 100 mM Potassium Phosphate buffer, **pH 7.85**, at room temperature.

B) 10 mM Nifedipine	Prepared by dissolving 7.0 mg nifedipine in **2.0 ml methanol** - store in ***amber vial*** and discard after use
C) 10 mM NADPH	Prepare by dissolving 4.7 mg in 0.5 ml Buffer A – enough for 20 assay tubes.
D) 1 M Sodium Carbonate	(Na_2CO_3) buffer, pH 10.5, containing 2 M NaCl.
E) Ethyl Acetate	
F) Methanol	
G) 1 mM Nifedipine Pyridine Metabolite (NPM)	7 mg NPM solid/20 ml of MeOH

II. PROCEDURE

1. Pipette appropriate amount of Reagent A 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 - 0.25 nmol microsomal P450) or purified reconstituted P450 (50 - 100 pmol) to the tubes.

3. Add 5 µl of Reagent B to each tube, giving a final nifedipine concentration of 200 µM.

4. Add 25 µl of Reagent C to the appropriate tubes, vortex, and place tubes in shaking water bath at 37°C. **DO NOT ADD** Reagent C to those incubation tubes that will be used as blanks or for standards.

5. Terminate the reactions after either 20 min (microsomes) or 10 min (purified P450s) by adding 50 µl of Reagent D to the tubes, vortexing the tubes well, and placing them on ice.

6. After all the reactions are stopped, add 1 ml of Reagent E to each tube, cap the tubes tightly, and vortex the tubes for 5 min using the Multiple Vortexer.

7. 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.

8. Add 60 µl of Reagent F to each tube, cover with a plastic cap, vortex briefly, and sonicate for 5 min in a bath-type sonicator to **completely** resolubilize residues. Process samples by HPLC as described below or store @ -20°C until HPLC analysis can be performed.

☞**NOTE A** - Blanks for this assay contain all components except NADPH (Reagent C). Standard curves are constructed by adding 2.5, 5, 10, and 15 µl of Reagent G (equivalent to 2.5, 5, 10, and 15 nmol nifedipine pyridine metabolite) to the assay tubes with NADPH OMITTED, and performing the assay as described above.

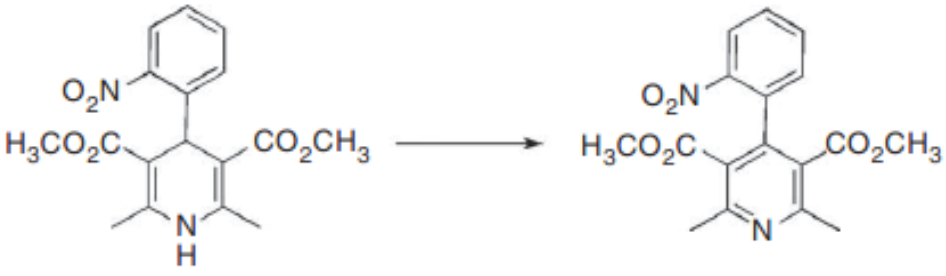
III. HPLC ANALYSIS CONDITIONS FOR NIFEDIPINE

Column:	Waters Sunfire (3.0 x 150 mm) w/ Security Guard STD Guard Column
Mobile Phase:	**Solvent A** – 0.05% Phosphoric Acid
	Solvent B – 100% MeOH
Flow Rate:	0.75 ml/min
Column Temp:	Ambient
Sample Temp:	Ambient
Peak Detection:	254 nm
Run Time:	16 min
Injection Volume:	30 µl

(min)	(%A)	(%B)	(ml/min)	
Initial	50	50	0.75	--
10	20	80	0.75	6
10.1	50	50	0.75	1

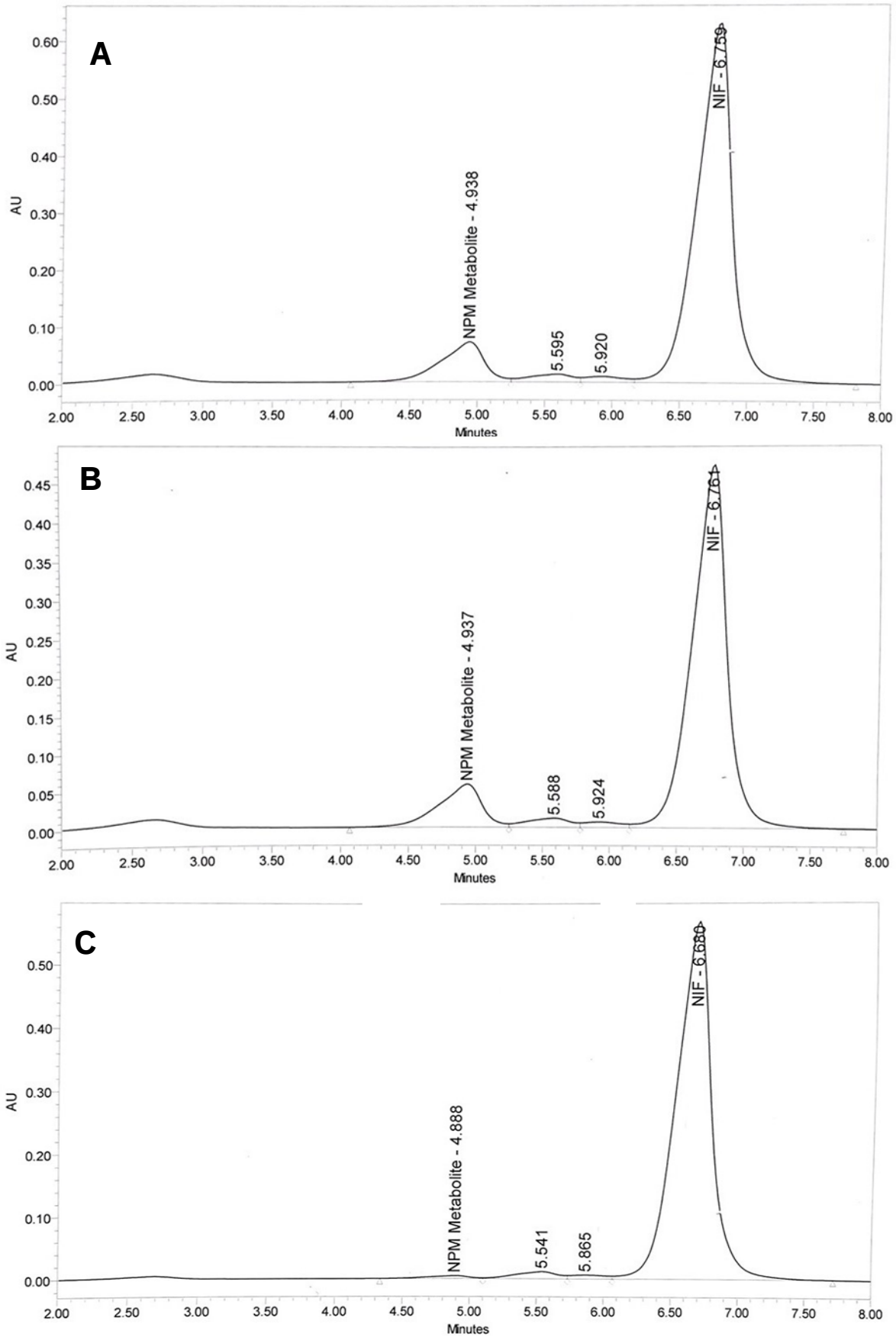
Using these HPLC conditions, NPM (nifedipine pyridine metabolite) elutes at **4.9 MIN** while the parent compound nifedipine elutes at **6.8 min** (see attached chromatogram).

REFERENCE: Guengerich FP *et al* : Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. *J. Biol. Chem.* 26:5051-5060, 1986.



NIFEDIPINE CONVERSION TO NIFEDIPINE PYRIDINE METABOLITE BY CYP3A4/CYP3A5
Nifedipine = 3,5-dimethyl 2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate.

NIFEDIPINE – CYP3A4



HPLC analysis of nifedipine oxidation by human liver microsomes. Nifedipine (NIF; 200 µM) was incubated with human liver microsomes (0.24 mg protein, 0.1 nmol aggregate P450) for 20 min at 37°C in the presence of NADPH plus 0.5 mg anti-CYP1A2, anti-CYP3A4 IgG or control (preimmune) IgG. NIF was then resolved from the nifedipine pyridine metabolite (NPM) metabolite formed 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 Waters Sunfire C18 column (3.0 mm x 15 cm, 5 µm particle size) was utilized. Gradient elution was performed with a mobile phase consisting of methanol:0.05% phosphoric acid (see above) employing a flow rate of 0.75 ml/min, and the column eluates were continuously monited for UV absorbance at 254 nm. Under these conditions, NPM and NIF exhibited retention times of 4.9 and 6.7 min, respectively; the total sample analysis time was 16 min. Formation of NPM was determined by comparison of peak areas with those of analytical metabolite standards.

a) NIF + liver microsomes + NADPH + Preimmune IgG
b) NIF + liver microsomes + NADPH + Anti-CYP1A2 IgG
c) NIF + liver microsomes + NADPH + Anti-CYP3A4 IgG