PHENACETIN *O*-DEETHYLASE ASSAY

This assay measures P450-catalyzed oxidation of the oral analgesic phenacetin **(PA)** to its *O*-deethylated metabolite acetaminophen (**APAP**), the latter of which is also an analgesic. High affinity phenacetin *O*-deethylation is catalyzed solely by CYP1A2, an inducible P450 enzyme present in human liver. Other P450s can convert PA to APAP but their affinity for this substrate is markedly less.

I. REAGENTS NEEDED

A) 100 mM Potassium Phosphate buffer, **pH 7.4**, at room temperature.

B) 5 mM Phenacetin (PA) — Stock 55.9 mM solution is prepared by dissolving 10 mg PA in 1.0 ml MeOH. Dilute 18 µl of this solution with 182 µl MeOH to give 5 mM PA. ***Buffer/PA solution is prepared by adding 80 µl 5 mM PA to 3.92 ml Reagent A*.** (enough for 18 assay tubes)

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

D) Acetonitrile

E) MeOH : 0.1% Acetic Acid (1:1, v/v)

E) 0.25 mM APAP
MW = 158.15 — Prepare by dissolving 8 mg APAP solid/2 ml MeOH to give **25** mM APAP. Dilute 50 µl of 25 mM APAP with 4.95 ml of MeOH to give 0.25 mM APAP. See

II. PROCEDURE

1. Pipette appropriate amount of Reagent A containing **100 µM PA** 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 human liver microsomes (0.05 - 0.1 nmol microsomal P450) to the tubes.

3. 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 incubation tubes that will be used as blanks or for standards.

4. Terminate reactions after **30 min** by adding 750 µl of Reagent D, and vortexing well.

5. Centrifuge tubes at 15,000 rpm for 10 min in the Heraeus Microfuge to precipitate protein. Transfer 700 µl of supernatant to a 10 x 75 mm disposable glass tubes, and evaporate sample to dryness at room temp using the N2-EVAP nitrogen evaporator. Because of water in the sample, evaporation will take 1.5 - 2 h.

6. Add **75** µl of Reagent E to each tube, 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** - Assay blanks contain all components except NADPH (Reagent C). Standard curves are constructed by adding 5, 10, 20 and 30 µl of Reagent E (equivalent to 1,25, 2.5, 5.0 and 7.5 nmol APAP) to the assay tubes with NADPH OMITTED, and performing assay as described above.

III. HPLC ANALYSIS CONDITIONS FOR APAP & PA

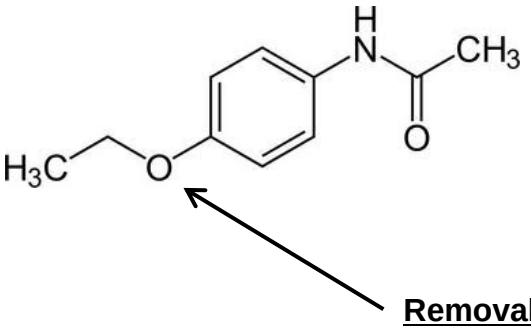
Column:	Waters Sunfire (3.0 x 150 mm) w/ Security Guard STD Guard Column
Mobile Phase:	**Solvent A** – 0.1% Acetic Acid
	Solvent B – ACN
Flow Rate:	0.75 ml/min
Column Temp:	Ambient
Sample Temp:	Ambient
Peak Detection:	**254 nm**
Run Time:	12 min
Injection Volume:	**30 µl**

Sunfire Column

(min)	(A %)	(B %)	(ml/min)	(gradient)
Initial	90	10	0.75	--
4.0	90	10	0.75	11
8.0	60	40	0.75	2
8.1	90	10	0.75	1

With these HPLC conditions, APAP elutes at **2.6 MIN** while the parent PA elutes at **7.9 MIN** (see attached chromatogram).

PHENACETIN [(N-(4-Ethoxyphenyl)acetamide]



Removal of ethyl group gives rise to APAP

REFERENCES

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von Moltke LL, Greenblatt DJ, Duan SX, Schmider J, Kudchadker L, Fogelman, SM, Harmatz JS, Shader, RI: Phenacetin *O*-deethylation by human liver microsomes in vitro: inhibition by chemical probes, SSRI antidepressants, nefazodone and venlafaxine. *Psychopharmacol* 128:398–407, 1996.

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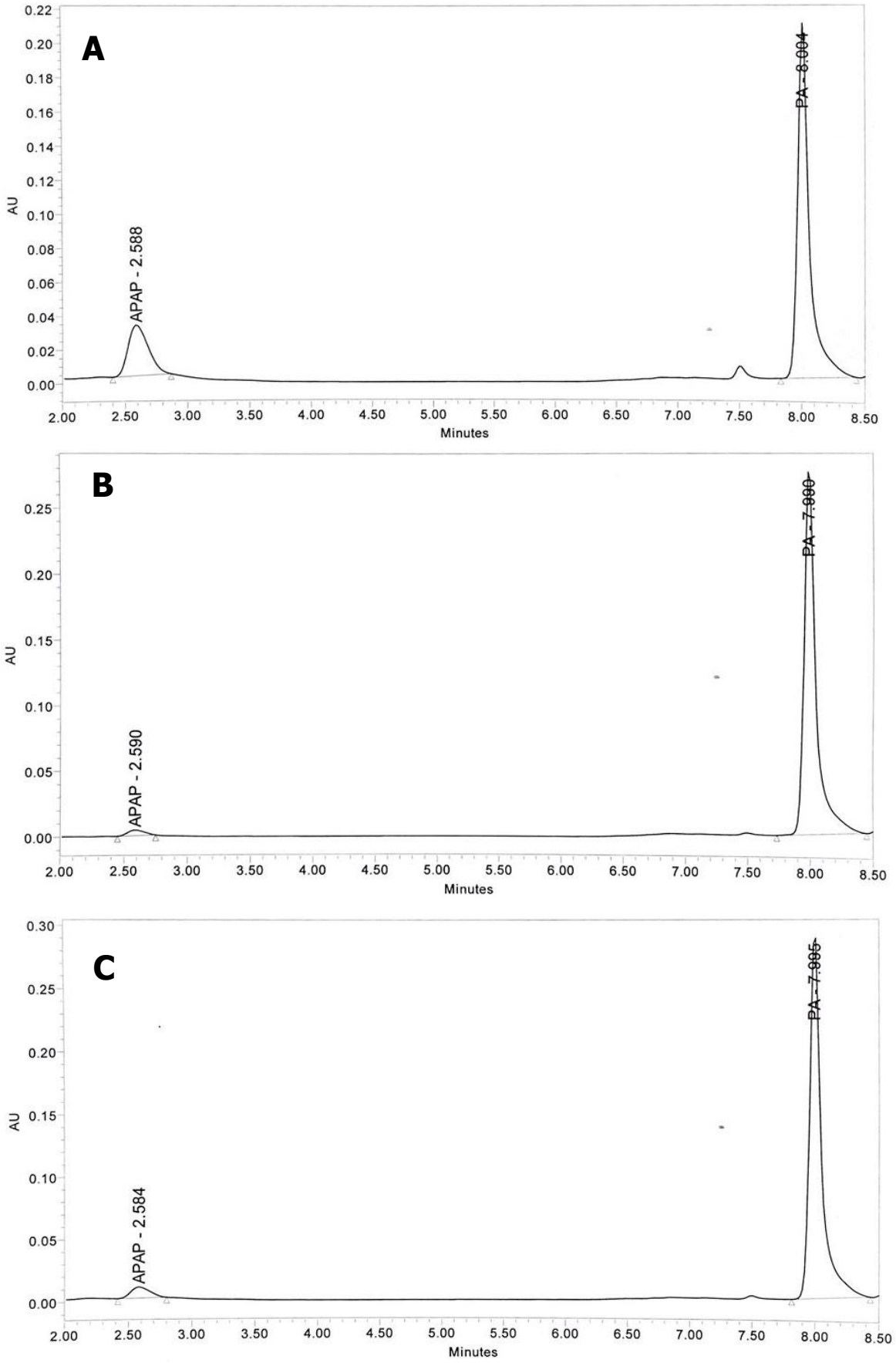
PHENACETIN – CYP1A2



HPLC analysis of phenacetin *O*-deethylation by human liver microsomes. Phenacetin (PA, 100 µM) was incubated with human liver microsomes (0.24 mg protein, 0.1 nmol aggregate P450) for 30 min at 37°C in the presence of NADPH plus 0.5 mg preimmune IgG, 0.05 mg anti-CYP1A2 mAb or 0.5 mg polyclonal anti-CYP1A2. PA was then resolved from the acetaminophen (APAP) 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 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 acetonitrile:0.1% acetic acid 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, APAP and PA exhibited retention times of 2.6 and 8.0 min, respectively; the total sample analysis time was 12 min. APAP formation was quantified by comparison of peak areas with those of analytical standards.

a) PA + liver microsomes + NADPH + Preimmune IgG
b) PA + liver microsomes + NADPH + Anti-CYP1A2 mAb
c) PA + liver microsomes + NADPH + Anti-CYP1A2 IgG