DEXTROMETHORPHAN *O*-DEMETHYLASE ASSAY

This assay measures the P450-dependent conversion of the antitussive agent dextromethorphan (DXM) to its *O*-demethylated metabolite dextrorphan (DXO). DXM *O*-demethylation occurs mainly in the liver, and is catalyzed exclusively by CYP2D6. CYP3A also metabolizes DXM but only at high substrate concentrations. Since CYP2D6 is a polymorphic P450 enzyme, metabolism of DXM exhibits genetic polymorphism. While most individuals display an extensive capacity to metabolize this drug (EM phenotype), 4-8% of all Caucasians hydroxylate DXM poorly (PM phenotype), which is due to a deficiency in hepatic CYP2D6 levels.

I. REAGENTS

A. 100 mM KPO_4 buffer, pH 7.4, at room temperature.

B. 2.5 mM DXM **MW = 370.3**	Prepared by dissolving **4.6 mg** DXM hydrobromide monohydrate in 5 ml of H_2O. Store at -20°C but sonicate before use (> thawing).
C. 10 mM NADPH	Prepare by dissolving 4.7 mg in 0.5 ml Buffer A – enough for 20 assay tubes.
D. Acetonitrile (ACN)	
E. MeOH	
F. 0.25 mM DXO MW = 267.1 (free acid)	Prepare by diluting 1 mg DXO/ml authentic std to 100 µg/ml with H2O [1 µg DXO = 3.7 nmol]

II. PROCEDURE

1. Prepare a mixture of 100 µM DXM in KPO_4 buffer by adding 200 µl of Reagent B to 4.8 ml of Reagent A (enough for 20 assay tubes).

2. Add the appropriate amount of the substrate/buffer mixture into 1.5 ml Eppendorf-type microfuge tubes, keeping in mind that the final assay volume is **0.25 ml**. Place tubes at 4°C.

3. Add human liver microsomes (0.1 - 0.2 nmol microsomal P450) to the tubes.

4. Add 25 µl Reagent C, vortex, and place tubes in shaking water bath at 37°C.

5. Reactions are terminated after **30 min** by adding 100 µl Reagent D, vortexing the tubes, and placing them in ice.

6. Centrifuge tubes at 15,000 rpm for 10 min in the Heraeus Microfuge to precipitate protein.

7. Transfer 125 µl of supernatant (do not to disturb pellets) to autosampler vials containing 150 µl glass inserts, seal with caps, and subject to HPLC or store @ -20°C until analysis is 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 to the assay tubes with NADPH OMITTED, and performing assay as described above.

III. HPLC ANALYSIS CONDITIONS FOR DXM & DXO

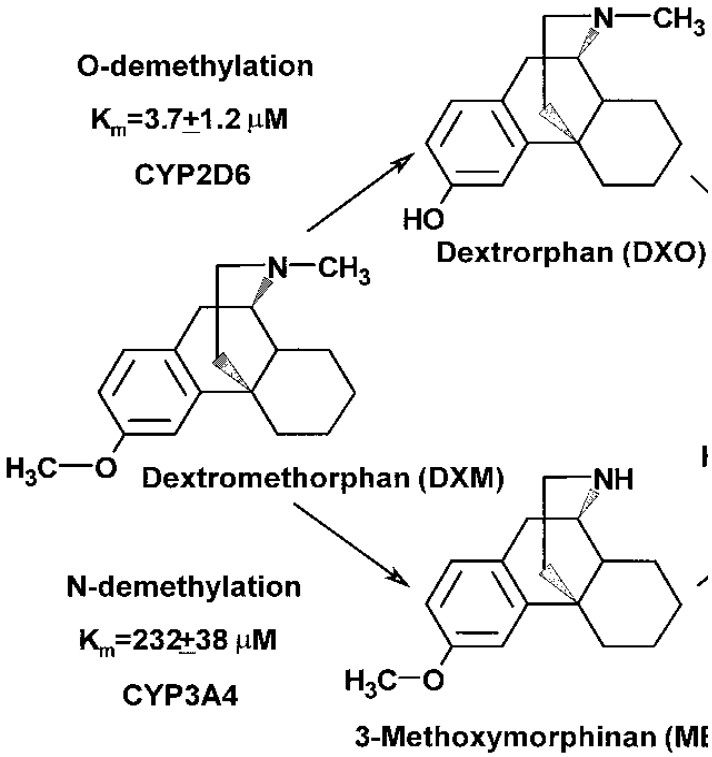
Column:	Phenomenex Kinetex column (4.6 x 150 mm) w/ Security Guard Column
Mobile Phase:	Solvent A – 0.05% Phosphoric Acid
	Solvent B – ACN
Flow Rate:	0.9 ml/min
Column Temp:	Ambient
Sample Temp:	Ambient
Peak Detection:	280 nm
Run Time:	16 min
Injection Volume:	50 µl

KINETEX Column

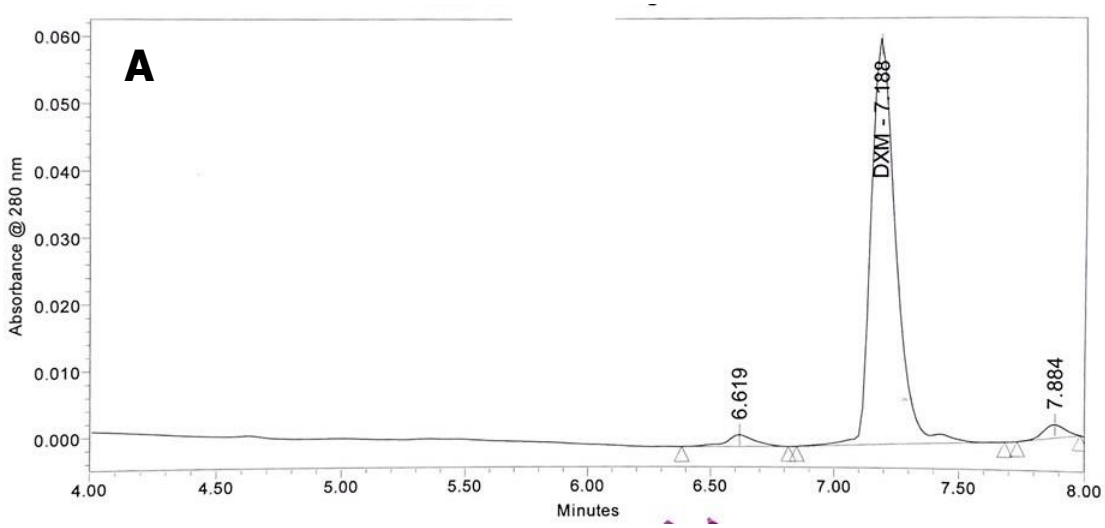
(min)	(% A)	(% B)	(ml/min)	(gradient)
Initial	90	10	1.0	--
10.0	40	60	1.0	6
10.1	90	10	1.0	1

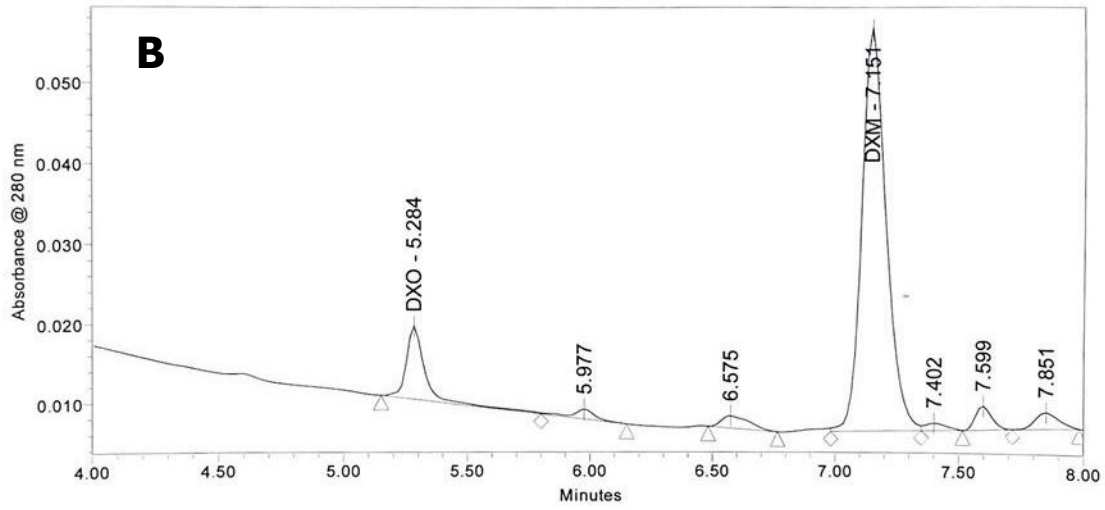
Using these HPLC conditions, DXO elutes at **5.2 MIN** while the parent compound DXM elutes at **7.2 min** (see attached chromatogram).

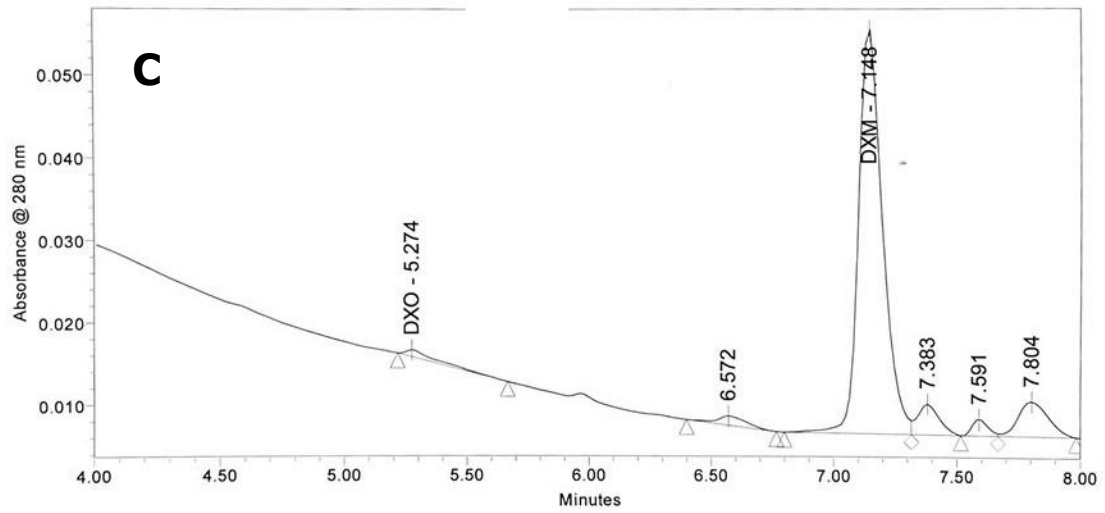
DEXTROMETHORPHAN *O*-DEMETHYLASE ASSAY



DEXTROMETHORPHAN – CYP2D6







HPLC analysis of dextromethorphan *O*-demethylase by human liver microsomes. Dextromethorphan (DMX; 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 immune-specific IgG or control (preimmune) IgG. DXM was then resolved from dextrorphan (DXO), the O-demethylated 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 was performed with a mobile phase consisting of acetonitrile:0.05% phosphoric acid (see above) employing a flow rate of 0.9 ml/min, and the column eluates were continuously monited for UV absorbance at 280 nm. Under these conditions, DXO and DXM exhibited retention times of 5.2 and 7.2 min, respectively; the total sample analysis time was 16 min. Formation of DXO was determined by comparison of peak areas with those of analytical metabolite standards.

a) DXM + liver microsomes + preimmune IgG (NADPH omitted)
b) DCF + liver microsomes + NADPH + Preimmune IgG
c) DCF + liver microsomes + NADPH + Anti-CYP2D6 IgG