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High-throughput fluorescence assay of cytochrome P450 3A4

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Abstract

Cytochrome P450 mono-oxygenases (P450s) are the principal enzymes involved in the oxidative metabolism of drugs and other xenobiotics. In this protocol, we describe a fluorescence-based, high-throughput assay for measuring the activity of P450 3A4, one of the key enzymes involved in drug metabolism. The assay involves the oxidative debenzoylation of a substituted coumarin, yielding an increase in fluorescence on reaction. The entire procedure can be accomplished in 1 h or less.

INTRODUCTION

Cytochrome P450 (P450) mono-oxygenases are heme-thiolate proteins that are found in almost all living organisms. In addition to the biosynthesis of essential endogenous molecules, such as steroids and eicosanoids, they are also responsible for the oxidative metabolism of a wide variety of xenobiotics^{1,2}. In all, 57 genes are found in humans, but five of the P450 enzymes—1A2, 2C9, 2C19, 2D6 and 3A4—are responsible for >90% of metabolism of drugs currently in clinical use²⁻⁴. Considerable emphasis is placed on the role of these P450s in the pharmaceutical industry, in terms of assays for contributions to metabolism of new chemical entities and enzyme inhibition.

Various *in vitro* assays for measuring the activity and inhibition of P450s have been developed over the years⁵. The most commonly applied method today is to use recombinant P450s with probe substrates, with the resulting products resolved by high-performance liquid chromatography (HPLC) and detected by a mass spectrometer (MS) coupled to the HPLC system⁶⁻⁸. These HPLC–MS based assays are highly sensitive and have been used as an industry standard method for P450 inhibition assessments. However, this method cannot be fit into a high-throughput format and may be problematic when large numbers of samples need to be tested (e.g., profiling the inhibitory potential of a chemical library against a specific P450 oxygenase). In this situation, fluorescence-based P450 assays are much faster and more cost effective than HPLC–MS assays^{9,10}. In these assays, P450 oxidizes a pro-fluorescent molecule to a fluorescent product. This product can be directly measured using a fluorescence microplate reader. However, all fluorescence measurements must be cautiously monitored for fluorescence interference or fluorescence quenching from tested compounds. In principle such assays can be carried out whenever the fluorescence of a reaction product is sufficient to be detected accurately. Examples include our work with P450 1A2 and resorufins¹¹ and P450 2A6 and coumarin 7-hydroxylation¹².

In this protocol, we present a microtiter plate-based fluorescence assay for the activity of P450 3A4. A number of coumarins and other fluorophores have been used in P450 fluorescence assays^{13–15}. 7-Benzoyloxy-4-trifluoromethyl coumarin (BFC) is used in this assay as the probe substrate and undergoes *O*-dealkylation to give the fluorescent product 7-hydroxy-4-trifluoromethyl coumarin (HFC, $\text{excitation } 405 \text{ nm} / \text{emission } 510\text{--}545 \text{ nm}$) (**Fig. 1**)¹⁵. In this protocol, we demonstrate how this assay can be applied in the determination of the IC₅₀ value of ketoconazole, a potent inhibitor of P450 3A4.

The literature contains fluorescent reactions that can be used with other P450s^{11–15}. Another alternative for high-throughput P450 activity assays is luminescence methods. Analogous to the fluorescence assays, a pro-luminescent substrate is used and yields a product that emits luminescence upon the addition of a developing agent¹⁶. The advantage of luminescence assays is the improved signal-to-noise ratio compared with fluorescence. The major drawback is that an additional development step is needed and the signal cannot be monitored in a continuous mode.

Experimental design

The protocol can be applied to directly determine IC₅₀ values for compounds that are potential P450 3A4 inhibitors. Recombinant P450 3A4 protein without other P450s is recommended in this assay, because other P450s, e.g., 1A2, can also transform BFC to HFC. The compounds to be studied should be dissolved in water if they are soluble. Otherwise, prepare the compounds in acetonitrile or methanol. Adjust the concentration of the inhibitor so that the organic solvent is < 1% (vol/vol) in the final reaction to avoid interference with P450 catalytic activities. The plate setup used in this protocol is described in **Table 1**. The reaction is carried out in duplicate in rows A and B. Varying amounts of ketoconazole are added to the wells (columns 1 through 10). Well 11 serves as the blank control, because the stopping buffer is added before the addition of the NADPH-regenerating system. The fraction of activity remaining with inactivated P450 3A4 (obtained in the presence of ketoconazole) compared with the activity in the absence of any added compound to the reaction mixture (column 12, control, absence of P450 3A4 inhibition) is used in the calculations. The ratio of inactivated P450 is plotted as a function of ketoconazole concentration using a 4-parameter logistic equation: $y = a + (b - a)/(1 + 10^{(x-c)d})$ using the software available at <http://www.changbioscience.com/stat/ec50.html>.

MATERIALS

REAGENTS

- Human P450 3A4 ‘bicistronic’ membranes¹⁷ containing both P450 and NADPH-P450 reductase (concentration of P450 is 1 μM). Similar preparations are available from BD Bioscience, cat. no. 456202.
- Potassium phosphate (Sigma-Aldrich, cat. no. P9791)
- NADP⁺ (Sigma-Aldrich, cat. no. N5755)
- Glucose-6-phosphate (Sigma-Aldrich, cat. no. G7879)
- Yeast glucose-6-phosphate dehydrogenase (Sigma-Aldrich, cat. no. G6378)
- Tris base (Sigma-Aldrich, cat. no. T1503)
- Methanol (Fisher, cat. no. A452-4) ! **CAUTION** Flammable and toxic. Use goggles and work in a fume hood.
- Acetonitrile (Fisher, cat. no. A998-4) ! **CAUTION** Flammable and toxic. Use goggles and work in a fume hood.

- BFC (BD Biosciences, cat. no. 451731)
- Ketoconazole (Sigma-Aldrich, cat. no. K1003)

EQUIPMENT

- 96-well black assay plate (Corning Costar, cat. no. 3915)
- Polarstar microplate reader (BMG Labtech)
- Microchannel pipetter (Gilson)
- Software to calculate IC₅₀ (<http://www.changbioscience.com/stat/ec50.html>)

REAGENT SETUP

Water for all buffers and incubations should be purified using a Milli-Q (Millipore, Billerica, MA, USA) system.

- NADPH-generating system: combine 50 parts 10 mM NADP⁺, 50 parts 0.1 M glucose-6-phosphate and 1 part 1 mg ml⁻¹ yeast glucose-6-phosphate dehydrogenase; prepare fresh daily; store on ice when not in use.
- 10 mM NADP⁺: 382 mg in 50 ml of (Milli-Q) water, remains stable for several months if it is stored at 4 °C.
- 0.1M glucose-6-phosphate: 3.4 g in 100 ml of (Milli-Q) water, remains stable for several months if it is stored frozen at -20 °C.
- 10³ IU ml⁻¹ yeast glucose-6-phosphate dehydrogenase: prepare at 1 mg ml⁻¹ in 10 mM Tris-acetate buffer (pH 7.4), containing 1.0 mM EDTA and 20% glycerol (vol/vol); it is to be stored at 4 °C. This remains stable for several months.
- 0.1M potassium phosphate buffer (pH7.4): 13.96 g dibasic potassium phosphate (Sigma-Aldrich cat. no. P288) and 2.69 g monobasic potassium phosphate (Sigma-Aldrich cat. no. P9791) per 1 liter (Milli-Q) water (add phosphate salts to water, not vice-versa); remains stable for several months at 4 °C.
- Stop buffer: 80% acetonitrile and 20% 0.5 M Tris-base (vol/vol), stored at an ambient temperature (23 °C); it remains stable for several months. **! CAUTION** Acetonitrile is flammable and toxic. Use goggles and work in a fume hood.
- 4 mM BFC: 2.56 mg in 2ml methanol, stored in a Teflon-sealed amber glass vial at 4 °C. This is stable for several months. **! CAUTION** Methanol is flammable and toxic. Use goggles and work in a fume hood.
- 1 mM ketoconazole: 5.31 mg in 10 ml methanol, stored in a Teflon-sealed glass vial at 4 °C. This is stable for several months. **! CAUTION** Methanol is flammable and toxic. Use goggles and work in a fume hood.
- 2× enzyme-substrate mix: mix P450 3A4 and BFC in 0.1 M potassium phosphate buffer so the final concentration of P450 is 20 nM and BFC is 40 μM. Prepare the mix fresh daily.

EQUIPMENT SETUP

- Configure the Polarstar reader to '**Standard and Time-Resolved Fluorescence**'. Install the appropriate excitation filters (405 nm in this study) and emission filter (445 nm in this study) in the filter wheels. Define the test method in the 'Well' mode.

PROCEDURE

Plate setup • TIMING 30 min

1. Dispense 0.06 ml of 0.1 M potassium phosphate buffer into each of the wells (columns 2–12) of a 96-well plate using a multichannel pipette.
2. Dispense 0.118 ml of 0.1 M potassium phosphate buffer and 0.002 ml of 1 mM ketoconazole into the wells in column 1.
3. Serially dilute 0.06 ml from the well (in column 1) to the other wells (columns 2–10). Remove the extra 0.06 ml in the well in column 10.
4. Dispense 0.1 ml of 2× enzyme–substrate mix in all the wells. Add 0.075 ml of stop buffer to the wells in column 11.
5. Incubate the plate in a 37 °C incubator for 5 min.
6. Remove the plate from the incubator and dispense 0.04 ml of NADPH-generating system into each well to initiate the reaction.

Plate incubation • TIMING 20 min

7. Incubate the plate for 15 min in a 37 °C incubator, and then add 0.075 ml of stop buffer to each well (except wells in column 11).

IC₅₀ calculation • TIMING 20 min

8. Scan the plate using the Polarstar plate reader, average the replicates of data for each column.
9. Subtract the blank from the mean value of all other columns and calculate the percent of inactivated enzyme for each dilution of ketoconazole (designated as I %): $I = (1 - (\text{mean of individual column} - \text{mean of column 11}) / (\text{mean of column 12} - \text{mean of 11})) \times 100$

TROUBLESHOOTING

10. Fit the data with the 4-parameter logistic fit

$$y = a + (b - a) / (1 + 10^{(x-c)d})$$

to determine the IC₅₀ using the free web-based software at TMDU Chemical Biology Database (Tokyo, Japan): <http://www.changbioscience.com/stat/ec50.html>.

TIMING

Steps 1–6, Plate setup: 30 min

Step 7, Plate incubation: 20 min

Steps 8–10, IC₅₀ calculation: 20 min

TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

ANTICIPATED RESULTS

Fluorescence readings from dealkylation of BFC by P450 3A4 and the data processing are shown in **Table 3**. Background fluorescence reading is shown in well 11, where the NADPH-generating system is added after the addition of stop buffer. The 100% enzyme activity value is obtained in well 12, where no inhibitor was added. Sometimes low signal/noise ratios (well 12/well 11 < 2) can be problematic. This is often caused by decreased P450 activities and can normally be corrected by increasing the enzyme concentration. The IC₅₀ determined by the 4-parameter logistic fitting was 54 nM, using the web-based program (**Fig. 2**).

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Figure 1.
O-Dealkylation of 7-benzoyloxy-trifluoromethyl coumarin by P450 3A4.

Figure 2.

Calculated IC₅₀ of ketoconazole on P450 3A4. Output from the free web-based program: <http://www.changbioscience.com/stat/ec50.html>. The ratio of inactivated P450 is plotted as a function of ketoconazole concentration using a 4-parameter logistic equation:

$$y = a + (b - a) / (1 + 10^{(x-c)d})$$

Plate setup: The concentration of ketoconazole (nM) in each well is listed. The row designations are from A to B, and the column designations are from 1 to 12.

TABLE 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	5000	2500	1250	625	312	156	78	39.1	19.5	9.76	Blank	No inhibitor
B	5000	2500	1250	625	312	156	78	39.1	19.5	9.76	Blank	No inhibitor

TABLE 2

Troubleshooting guide for P450 3A4 fluorescence assay.

Step number	Problem	Possible reason	Solution
9	Low signal/noise ratio	Inactive NADPH-generating system	Verify that the NADPH-generating system is working
		Decreased P450 activity	Increase the concentration of P450 in the reaction
	Inconsistent amounts of product formation between replicates	Failure to equilibrate the temperature of plates before initiating the reaction	Pre-incubate plate for 5 min at 37°C

TABLE 3

Fluorescence readings and IC₅₀ calculation of the P450 3A4 inhibitor ketoconazole.

	1	2	3	4	5	6	7	8	9	10	11	12
A	7845	8072	8229	8729	9536	11753	15018	18213	22589	23408	7489	24886
B	7571	7647	7850	8861	9827	10940	14914	16373	20459	21789	7223	23760
Mean	7708	7859	8039	8795	9681	11346	14966	17293	21524	22598	7356	24323
Mean-Blank	424	503	683	1439	2325	3990	7610	9937	14168	15242	0	16967
I%	97.5	97	96	91.5	86.3	76.5	55.1	41.4	13.8	9.1		
Inhibitor concentration (nM)	5000	2500	1250	625	312	156	78	39.1	19.5	9.76		

These fluorescence readings were obtained using ketoconazole as an inhibitor of P450 3A4. Concentrations of ketoconazole (nM) 5000, 2500, 1250, 625, 312, 156, 78, 39.1, 19.5 and 9.76 in columns 1–10, respectively. Column 11 blank and column 12 no inhibitor.