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High-throughput fluorescence assay of cytochrome P450 3A4

Qian Cheng and F. Peter. Guengerich

Summary

Microtiter plate-based fluorescence assays allow rapid measurement of the catalytic activities of cytochrome P450 oxygenases (P450s). We describe a high-throughput fluorescence assay of P450 3A4, one of the key enzymes involved in xenobiotic metabolism. The assay involves the oxidative debenzoylation of 7-hydroxy-4-trifluoromethyl coumarin, producing an increase in fluorescence.

Keywords

P450; fluorescence; high-throughput assay; enzyme inhibition

1. Introduction

Cytochrome P450 oxygenases (P450s) are the major enzymes involved in the oxidative metabolism of drugs and other xenobiotics (1). Inhibition of P450s catalytic activities is a principal mechanism for *in vivo* drug-drug interactions (2–4). Various *in vitro* assays have been developed to measure the activity or inhibition of P450s (5). Historically, the most commonly applied method is to use recombinant P450s with probe substrates, with the products identified by high-performance chromatography (HPLC) coupled with mass spectrometry (MS) (6–8). However, this method is relatively time-consuming and labor intensive and cannot be readily adapted to high-throughput formats (e.g. testing the inhibitory properties of a chemical library). Thus, microtiter plate-based fluorescence assays are more efficient and cost effective than HPLC-MS methods, allowing rapid *in vitro* testing of many samples in parallel (9, 10), if they provide accurate reports of the function of the enzymes. In a fluorescent assay, a “pro-fluorescent” molecule is oxidized by P450 to a fluorescent product, which can be directly measured using a fluorescence microplate reader. P450 3A4 is, in most individuals, the most abundant P450 in the liver and small intestine and is involved in the metabolism of about one-half of the drugs on the market. Here we describe a high-throughput fluorescence assay for measuring the activity of P450 3A4. A number of pro-fluorescence substrates (mainly benzyl ethers) have been used in P450 3A4 fluorescence assays (11, 12). 7-Benzoyloxy-4-trifluoromethyl coumarin (BFC) is selected in this protocol because BFC metabolism by P450 3A4 is linear with respect to BFC concentration up to 100 μ M (13). A typical P450 3A4 inhibitor, ketoconazole, is used to demonstrate the use of microtiter plate-based fluorescence assays to assess the activity of P450 3A4 and characterize the IC₅₀ of this inhibitor.

2. Materials

2.1 P450 assay

1. Human P450 3A4 'bicistronic' membranes (14) containing both P450 3A4 and NADPH-P450 reductase (concentration of both enzymes is 1 μ M). Similar commercial products are available from BD Biosciences (see Note 1).
2. 10 mM NADP⁺ stock solution: 382 mg NADP⁺ in 50 ml Milli-Q water. Store at 4 °C.
3. 100 mM glucose-6-phosphate: 3.4 g in 100 ml Milli-Q water. Store at 4 °C.
4. 10³ IU/ml yeast glucose-6-phosphate dehydrogenase: 1.0 mg in 1 ml 10 mM Tris-acetate buffer (pH 7.4), containing 20% glycerol (v/v) and 1.0 mM EDTA. Store at 4 °C.
5. 4 mM BFC stock solution: 2.56 mg in 2 ml methanol. Stored in Teflon-sealed amber glass vial at 4 °C.
6. 1 mM ketoconazole stock solution: 5.31 mg in 10 ml methanol. Stored in Teflon-sealed glass vial at 4 °C.
7. NADPH-generating system: combine 50 parts 10 mM NADP⁺, 100 parts 100 mM glucose-6-phosphate and 1 part 1 mg/ml yeast glucose 6-phosphate dehydrogenase. Prepare fresh daily and store on ice when not in use (see Note 2).
8. Stop buffer: 80% acetonitrile and 20% 0.5 M Tris-base (v/v), stored at ambient temperature.
9. P450-BFC 2 $\times$ mix: mix P450 3A4 bicistronic membranes and 4 mM BFC stock solution in 100 mM potassium phosphate buffer (pH 7.4) so that the final concentration of P450 is 20 nM and BFC is 40 μ M. Prepare the mix fresh daily and keep on ice.

2.2. Equipment

1. Microplate reader. Polarstar microplate reader is used in this protocol (BMG Labtech)
2. 96-well black microtiter plate (Corning Costar)
3. Microchannel pipette (Gilson)
4. Software to process the data. Prism (Graphpad.com) is used in this protocol (see Note 3).

¹The activities of P450 3A4 'bicistronic' membranes may decrease over the time. Avoid extensive thaw-freeze cycles.

²Determine that the NADPH-generating system is active by monitoring the increase in absorbance at 340 nm.

³Other statistical software (than GraphPad Prism) can perform nonlinear regression can be used for this experiment. Web-based curve fitting programs are also available, e.g. www.chanbioscience.com/stat/ec50.html.

3. Methods

3.1 Plate setup

The assay is performed in duplicate and the plate setup is summarized in Table 1.

1. Dispense 60 μ l 100 mM potassium phosphate buffer into the wells (column 2–12) in a 96-well microplate using a multichannel pipette.
2. Dispense 118 μ l 100 mM potassium phosphate and 2 μ l of 1 mM ketoconazole into the wells (column 1).
3. Serially dilute 60 μ l inhibitor solution from the wells in column 1 to the other wells (2–10). Discard the extra 60 μ l solution in the wells in column 10.
4. Dispense 100 μ l P450-BFC 2 $\times$ mix in all the wells. Add 75 μ l stop buffer to the wells in column 11.
5. Preincubate the plate at 37 °C for 5 minutes ((see Note 4).
6. Add 40 μ l NADPH generating system into each well to initiate the reaction.
7. Incubate the plate at 37 °C for 20 minutes.
8. Stop the reaction by adding 75 μ l stop buffer to each well (except wells in column 11).

3.2 Data acquirement and processing

1. Scan the plate using the Polarstar plate reader. Average the replicates of the reading for each column.
2. Subtract the blank from the mean value of all other columns and calculate the percentage of inactivated enzyme for each ketoconazole concentration (designated as I%). $I = (1 - (\text{mean of individual column} - \text{mean of column 11}) / (\text{mean of column 12} - \text{mean of column 11})) \times 100$.
3. Convert the concentration of inhibitor into log format (Designated as X).
4. Fit the data with the 4-parameter logistic fit: $I = b + (a - b) / (1 + 10^{((\text{LogIC}_{50} - X) \times \text{HillSlope}))}$ using Prism. (Note 5)
5. The processed data is shown in Table 2 and fitted plot is shown in Figure 1.
6. Subtract the blank from the mean value of all other columns and calculate the percentage of inactivated enzyme for each ketoconazole concentration (designated as I%). $I = (1 - (\text{mean of individual column} - \text{mean of column 11}) / (\text{mean of column 12} - \text{mean of column 11})) \times 100$.
7. The calculated log IC₅₀ is 1.57 to 1.92. Thus the measured IC₅₀ in this experiment is 37–83 nM. (Note 6)

⁴Preincubation prior to the addition of NADPH to equilibrate temperature is a critical step. Insufficient preincubation can result in inconsistent readings between replicates.

⁵Although simple linear regression may be used in certain situation, the 4-parameter logistic fit is more appropriate in most cases. Parameter **a** is the highest response and **b** is the lowest response.

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⁶The raw data used in this protocol was reported previously (15).

Figure 1.

Calculated IC_{50} of ketoconazole with P450 3A4. The response (inhibition percentage) is plotted against the $\log_{10}$ of ketoconazole concentration.

The scheme of plate setup: The assay are performed in duplicated as designated by row I and row II. The concentration of ketoconazole (nM) in each well is listed.

Table 1

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

Fluorescence reading of each well and data processing. Data listed in bold font is used for 4-parameter logistic fit: $I = b + (a - b) / (1 + 10^{-(\text{LogIC}_{50} - X) \times \text{HillSlope}})$.

Table 2

	1	2	3	4	5	6	7	8	9	10	11	12
I	7845	8072	8229	8729	9536	11753	15018	18213	22589	23408	7489	24886
II	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		
X	3.7	3.4	3.1	2.8	2.5	2.2	1.9	1.6	1.3	1		