Chapter 23

Assay of Endocannabinoid Oxidation by Cytochrome P450

Natasha T. Snider, Vyvyca J. Walker, and Paul F. Hollenberg

Abstract

Cytochrome P450 enzymes are a large family of heme-containing proteins that have important functions in the biotransformation of xenobiotics, including pharmacologic and environmental agents, as well as of endogenously produced chemicals with broad structural and functional diversity. Anandamide and 2-arachidonoylglycerol (2-AG) are substrates for P450s expressed in multiple tissues, leading to the production of a diverse set of mono- and di-oxygenated metabolites. This chapter describes tools and methods that have been used to identify major endocannabinoid-metabolizing P450s and their corresponding products, by using subcellular tissue fractions, cultured cells, and purified recombinant enzymes in a reconstituted system.

Key words P450 epoxygenase, P450 hydroxylase, Metabolism, Oxidation, Microsomes, Liquid chromatography-mass spectrometry, Eicosanoid

1 Introduction

Due to their structural similarity to arachidonic acid, the endocannabinoids anandamide and 2-AG are substrates for the three major classes of eicosanoid-metabolizing enzymes like cyclooxygenases, lipoxygenases, and cytochrome P450 [1–3]. The P450 branch of endocannabinoid metabolism yields a vast number of hydroxylated and epoxygenated products in vitro [4–9]. Furthermore, the epoxides of anandamide are subject to secondary metabolism via P450-mediated hydroxylation and epoxide hydrolase-mediated hydrolysis [6] (Fig. 1).

The 5,6-EET-EA metabolite of anandamide has increased stability compared to anandamide itself, and binds to type-2 cannabinoid (CB_2) receptor with nanomolar affinity, leading to decreased cAMP production in CB_2-expressing cells [10]. Murine BV-2 microglial cells stimulated with the pro-inflammatory cytokine interferon-γ (IFNγ) upregulate P450 3A1, leading to increased production of 5,6-EET-EA when the cells are treated with anandamide [10]. Similarly, the 2-AG epoxides 2-11,12-epoxyeicosatrienoylglycerol (EG) and 2-14,15-EG, which are generated by P450

Mauro Maccarrone (ed.), *Endocannabinoid Signaling: Methods and Protocols*, Methods in Molecular Biology, vol. 1412,
DOI 10.1007/978-1-4939-3539-0_23,

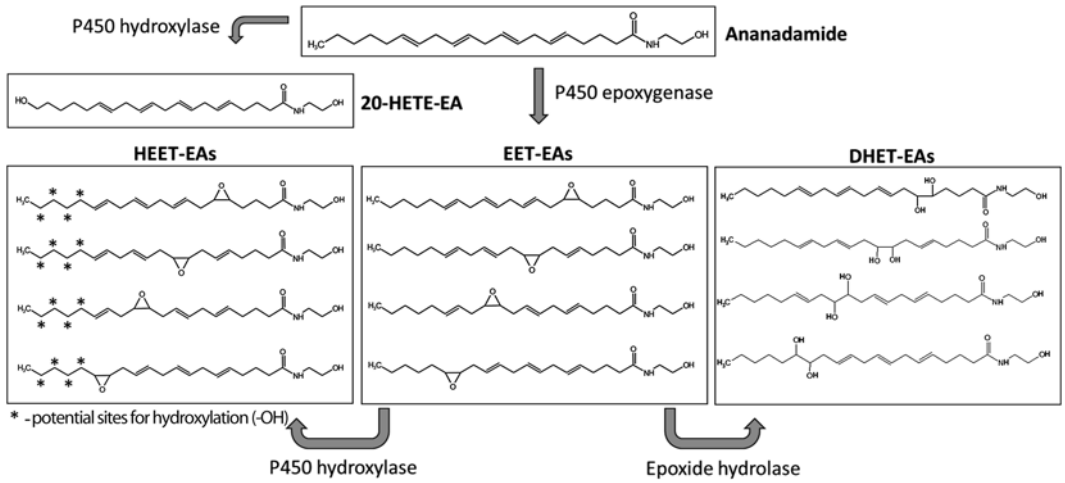

Fig. 1 Cytochrome P450 and epoxide hydrolase-derived anandamide metabolites. *HETE-EA* hydroxyeicosatetraenoic acid ethanolamide, *EET-EA* epoxyeicosatrienoic acid ethanolamide, *HEET-EA* hydroxy-epoxyeicosatrienoic acid ethanolamide, *DHET-EA* dihydroxyeicosatrienoic acid ethanolamide

epoxygenases, are ligands for the cannabinoid receptors, and are able to trigger downstream signaling events in cells (ERK signaling activation and cell migration) and physiological responses in mice (hypomotility, hypothermia, vasorelaxation) [11, 12]. Therefore, at least some of the P450-generated products of anandamide and 2-AG may be components of bioactivation pathways under specific contexts. Given that endocannabinoids are produced on demand and their concentrations are not easily quantified in tissues, studying the fate and functions of their metabolic products in vivo is a challenging task. Therefore, the physiological relevance of endocannabinoid metabolites generated by P450s, as well as by other endocannabinoid oxygenases, remains to be fully determined. In vitro P450 metabolism assays are useful to determine the contribution of specific P450 isoforms, thus elucidating the structural identity and kinetic parameters of substrate formation, and understanding the signaling pathways that may alter the P450 branch of endocannabinoid metabolism.

The three major tools to carry out in vitro metabolic assays of endocannabinoids are tissue microsomes (endoplasmic reticulum membranes enriched in P450s), purified P450s, and P450-expressing cells in culture. In general, the metabolic products are separated and analyzed by using liquid chromatography coupled with mass spectrometry (Fig. 2). Since P450 isoform-specific expression and activity change dynamically in response to (patho) physiological conditions, tissue-derived microsomes represent a valuable tool for investigating context-specific metabolic changes. Primary cells or cell lines are well suited for mechanistic studies, whereas purified enzymes are critical for performing enzyme kinetics and characterization of specific metabolites.

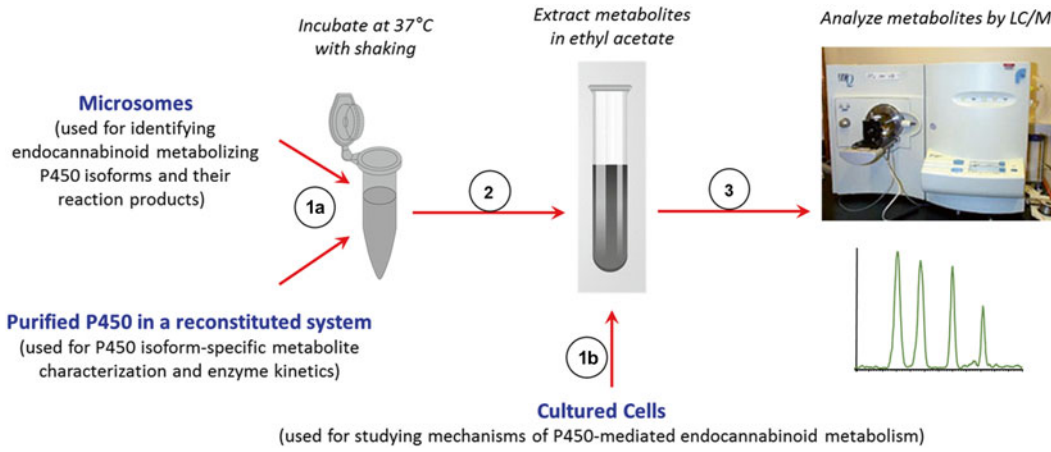

Fig. 2 General workflow for studying P450-mediated endocannabinoid metabolism in vitro

2 Materials

Microsomes should be prepared from freshly harvested tissues whenever possible, in order to minimize variability in the preparations [13]. In addition to microsomes, brain mitochondria are also enriched in P450s [14], so we recommend to analyze also this fraction in the context of brain P450s. Other sources of P450 enzymes are primary cells (most commonly hepatocytes) or recombinant enzymes that are used in a reconstituted system (*see* **Note 1**). Microsomes prepared from different human and animal tissues (generally in a pooled format), primary hepatocytes, and purified P450s are available from numerous commercial sources that are specialized in drug metabolic products (*see* **Note 2**).

2.1 Components for Metabolic Reactions

1. Source of P450: Tissue microsomes, cells, or purified P450.
2. Homogenization buffer for subcellular fractionation: 0.32 M Sucrose, 50 mM KH_2PO_4, 1 mM EDTA, 0.1 mM dithiothreitol, pH 7.4.
3. Microsomal buffer: 100 mM KH_2PO_4, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20 % (vol/vol) glycerol, pH 7.4.
4. 100 mM Potassium phosphate (KPO_4) buffer, pH 7.4: Make 1 M K_2HPO_4 (dissolve 174.18 g in 1 l of water) and 1 M KH_2PO_4 (dissolve 136.09 g in 1 l of water). Mix 80.2 ml of 1 M K_2HPO_4 with 19.8 ml of 1 M KH_2PO_4, and dilute the mixture to 1 l with water to obtain 100 mM potassium phosphate buffer at pH 7.4.
5. Endocannabinoid substrate (anandamide or 2-AG): Prepare stock solutions by using nitrogen-purged ethanol, and store at −80 °C in tightly capped silanized glass vials. Stocks should be used within 4 weeks.

6. NADPH (nicotinamide adenine dinucleotide phosphate) cofactor: To make a 50 mM stock solution dissolve 42 mg β-NADPH in 1 ml of water. NADPH solutions should be prepared fresh just before use, and should be kept on ice. Use stocks at 1 mM final concentration in reaction mixtures.
7. Catalase: From bovine liver, use at 50 U per reaction for use in the reconstituted system (*see* **Note 3**).
8. Lipid mixture for reconstitution of purified P450s: Use 10 μg per reaction of a 1:1:1 mixture of L-α-dilauroyl-phosphocholine, L-α-dioleyl-*sn*-glycero-3-phosphocholine, and L-α-phosphatidylserine.
9. P450 reductase: Purified recombinant enzyme, use at 50 pmol per reaction. For use in the reconstituted system.
10. Epoxide hydrolase: Purified recombinant enzyme, use at 1:1 ratio to P450.
11. P450 inhibitors: Broad and isoform-selective chemical and antibody inhibitors for various P450s are widely available from multiple commercial sources.

2.2 Components for Metabolite Extraction and Analysis

1. Ethyl acetate.
2. Methanol.
3. Acetic acid.
4. Deuterated standards for quantitation: Deuterated anandamide or 2-AG. All stock solutions, prepared using nitrogen-purged ethanol, should be stored in tightly capped silanized glass vials at −80 °C, and should be used within 4 weeks.
5. EET-EA and HETE-EA standards for generating standard curves (*see* **Note 4**).
6. Reversed-phase liquid chromatography column.
7. HPLC system.
8. Solvent A: 0.1 % Acetic acid in water.
9. Solvent B: 0.1 % Acetic acid in methanol.
10. Mass spectrometer.

3 Methods

3.1 General Protocol for the Preparation of Tissue Mitochondria and Microsomes

1. Fresh or frozen tissue samples are placed on ice in 2–4 volumes of homogenization buffer (0.32 M sucrose, 50 mM KH_2PO_4, 1 mM EDTA, 0.1 mM dithiothreitol, pH 7.4), and are homogenized (100 strokes) to a suspension by using a Potter-Elvehjem polytetrafluoroethylene (PTFE) pestle and glass tube homogenizer. It is best to start with at least 0.25–0.50 g tissue, which may require pooling of small-size samples (e.g., patient-derived material).

2. The starting tissue homogenate is centrifuged for 8 min at 1500 × *g*, the pellet is discarded, and the supernatant is collected and centrifuged again for 20 min at 12,000 × *g*.
3. After an additional wash in homogenization buffer and centrifugation for 20 min at 12,000 × *g*, the pellet from **step 2** is resuspended in 2–4 volumes of homogenization buffer (this is the mitochondrial fraction).
4. The supernatant from **step 2** is centrifuged at 105,000 × *g* for 75 min using a table-top or preparative ultracentrifuge.
5. The supernatant is discarded and the pellet from **step 4** is washed once in microsomal buffer (100 mM KH_2PO_4, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20 % (vol/vol) glycerol, pH 7.4), and centrifuged again for 75 min at 105,000 × *g*. The washed pellet is resuspended in buffer B (this is the microsomal fraction).
6. Protein concentration of the mitochondrial and microsomal fractions is measured using a reducing agent-compatible protein assay kit.
7. Protein samples are analyzed for the presence of specific organelle marker proteins (e.g., mitochondrial cytochrome c and microsomal cytochrome P450 reductase) by Western blot, as a quality control of the fractionation procedure.
8. The fractions are aliquoted and stored at –80 °C for long-term use in metabolic reactions.

3.2 Metabolic Reactions

1. *Microsomes*: Incubation mixtures generally contain 75–100 μg microsomal protein, endocannabinoid substrate (0.1–100 μM dose range), and 100 mM KPO_4 buffer pH 7.4 in a final volume of 0.5 ml. The reactions are incubated for 10 min (*see* **Note 5**), and are initiated by the addition of 1 mM NADPH. A negative control (without NADPH) should always be included. Prior to the addition of NADPH, microsomes may be preincubated for 5 min with chemical or antibody inhibitors, in order to assay the relative contribution of specific P450 isoforms to endocannabinoid metabolism (*see* **Note 6**).
2. *Cells in culture*: For cell-based studies endocannabinoid substrate (20 μM starting concentration) in serum-free medium is added to the cells (plated onto 10 cm dishes), and incubation follows for 45 min (*see* **Note 7**).
3. *Purified P450s*: For metabolic reactions with purified P450s in an in vitro-reconstituted system mixtures contain 25–50 pmol of purified enzyme, 2 molar excess of P450 reductase, 10 μg of lipid mixture, and 50–500 U of catalase in 100 mM potassium phosphate buffer. After 45-min incubation on ice, substrate and NADPH are added, and the reactions are stopped after

10 min (*see* **Note 8**). To assay secondary metabolism of epoxides, purified recombinant microsomal epoxide hydrolase may be added to the reconstitution reaction during the 45-min incubation, prior to the addition of substrate and NADPH.

3.3 Extraction of Metabolites and Known Standards

1. After incubations are completed, all reactions containing either microsomal or purified P450s are spiked with 100 pmol of deuterated endocannabinoid standard (internal control for extraction efficiency), are immediately stopped by the addition of 4 reaction volumes (2 ml) of nitrogen-purged ethyl acetate, and are vortexed for 1–2 min.
2. Cultured cells are scraped into their medium and subjected to several freeze-thaw cycles to induce lysis. Samples are spiked with deuterated internal standard and extracted as above.
3. For quantitation studies, standard curves are generated by extracting known amounts of synthetic standards (EET-EAs and HETE-EAs) from the same matrix components as the reactions (e.g., potassium phosphate buffer or serum-free culture medium).
4. Samples are centrifuged at $1200 \times g$ to separate the top organic ethyl acetate layer from the bottom aqueous layer.
5. The organic layer is carefully collected, dried under a gentle stream of nitrogen, and resuspended in 100 μl of 100 % methanol (*see* **Note 9**).

3.4 Liquid Chromatography-Mass Spectrometry Analysis

1. Samples are loaded onto autosampler vials, and 10 μl is injected onto the appropriate reversed-phase column. We use a 4.6×100 mm Hypersil ODS column, 5 μm particle size (Thermo Scientific).
2. The column is pre-equilibrated with 75 % Solvent B and 25 % solvent A. Metabolites are resolved through the following gradient: 0–5 min, 75 % B; 5–20 min, 75–100 % B; 20–25 min, 100 % B; 25–26 min, 100–75 % B; and 26–30 min, 75 % B. The flow rate is 0.3 ml/min.
3. The mass spectrometer conditions for anandamide metabolism on an LCQ mass analyzer (Thermo Scientific) are as follows: sheath gas, 90 U; auxiliary gas, 0 U; capillary temperature 200 °C, and spray voltage 4.5 V. Data are acquired in positive ion mode by using one full scan from 300 to 500 mass-to-charge (m/z) ratios and one data-dependent scan of the most intense ion.

3.5 Data Analysis

1. Typical metabolic data using anandamide as substrate are shown for several purified P450s (Fig. 3), BV-2-cultured microglia cells (Fig. 4), and human liver microsomes (Fig. 5).
2. Using running conditions as specified above, m/z ratios of anandamide, EET-EAs, HETE-EAs, DHET-EAs, and HEET-EAs are 348, 364, 364, 382, and 380. The elution order is as follows:

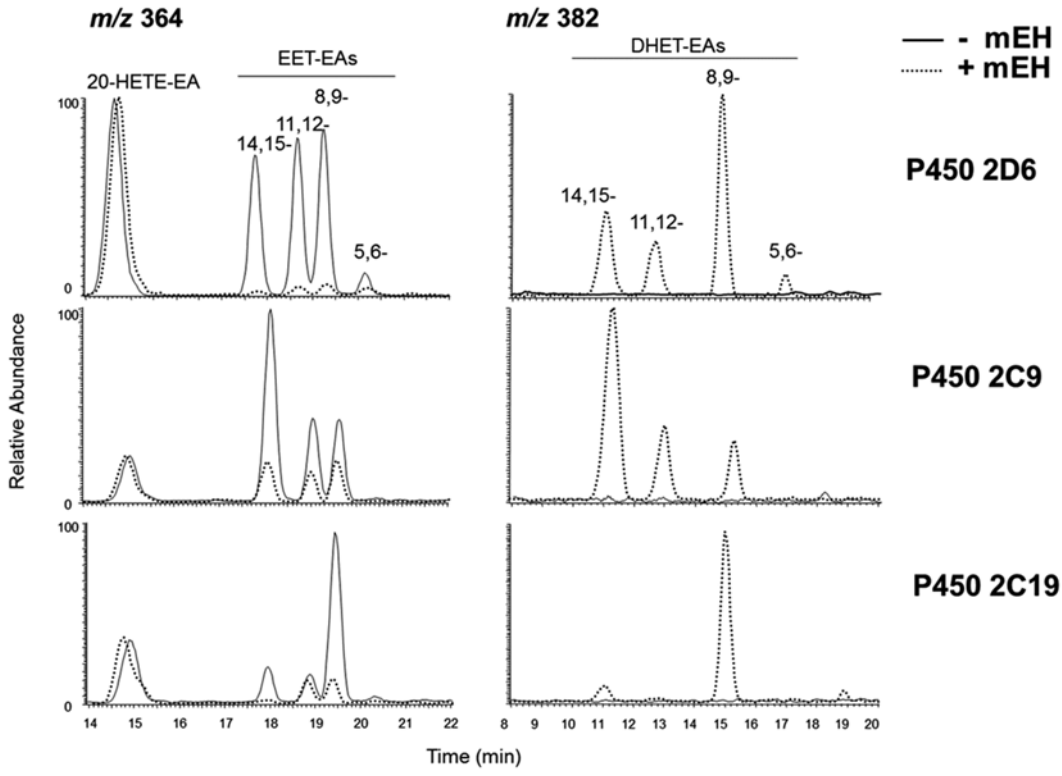

Fig. 3 Metabolism of anandamide by purified P450s 2D6, 2C9, and 2C19 in the reconstituted system, in the presence and absence of microsomal epoxide hydrolase. *Left* panels show the mono-oxygenated HETE- and EET-EAs (note the different metabolic profiles depending on the enzyme). *Right* panels show the di-oxygenated DHET-EAs, generated in the presence, but not in the absence, of microsomal epoxide hydrolase (mEH)

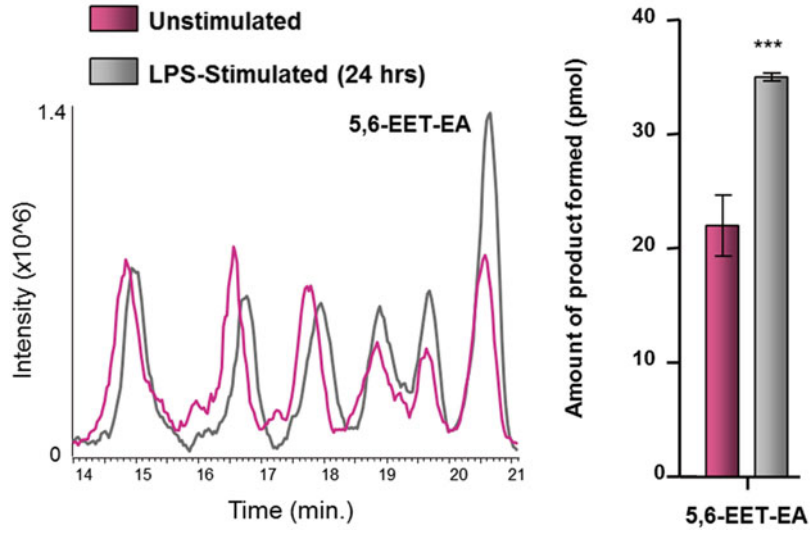

Fig. 4 P450 metabolites of anandamide formed by murine microglial BV-2 cells. The cells were either unstimulated or treated with lipopolysaccharide (LPS, 0.1 μg/ml for 24 h). Note the increased conversion of exogenously added anandamide to 5,6-EET-EA after LPS stimulation

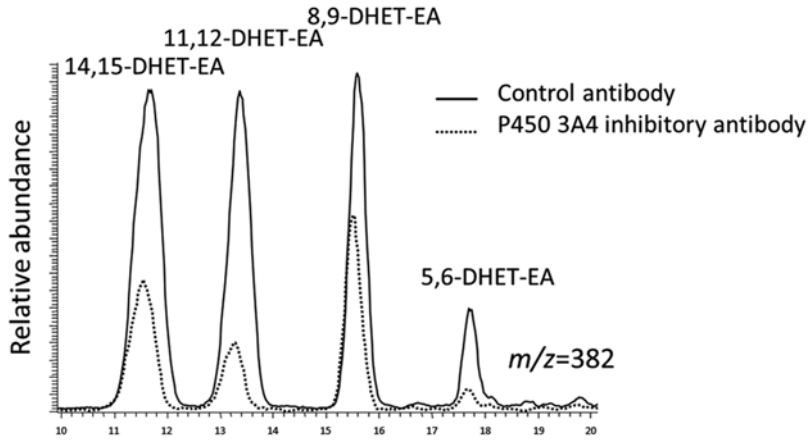

Fig. 5 Metabolism of anandamide by human liver microsomes in the presence and absence of an inhibitory antibody to P450 3A4. Shown are the di-oxygenated DHET-EA products, generated by microsomal epoxide hydrolase metabolism of the corresponding EET-EAs (extracted ion chromatogram $m/z = 382$)

HEET- EAs between 7 and 14 min; DHET-EAs between 11 and 18 min; 20-HETE-EA at 15 min; EET-EAs between 17 and 21 min; and the parent compound anandamide at 23 min.

3. Metabolite peak areas can be integrated by using an appropriate software (Xcalibur in our laboratory), and are expressed as ratios to the internal standard (d8-anandamide, m/z 356) in order to check extraction efficiency.
4. Peak areas of the synthetic metabolite standards (also normalized to an internal control) are similarly integrated to generate a standard curve (*see* **Note 10**).

4 Notes

1. Please note that primary cells should be used whenever possible, since most immortalized cell lines have generally low basal P450 expression and activity, as well as limited potential for P450 induction [15].
2. There is no standardized method for preparing tissue microsomes, and commercially available hepatic microsome preparations vary significantly in their P450 content and activity: this is a major *caveat* for these studies. We recommend to begin with a general subcellular fractionation protocol and to further optimize the specific buffer conditions. Although starting with fresh tissue is ideal, microsomes can also be prepared from snap-frozen human or animal tissues that have been properly stored for short or long term in liquid nitrogen [16].

3. Catalase is added to prevent inactivation of P450s by hydrogen peroxide. For P450 2D6 and P450 Supersomes™ assays, we add 500 U catalase. Supersomes™ Corning are commercially available microsomes from baculovirus-infected insect cells that express a specific P450 enzyme.
4. Synthetic standards for most P450-generated endocannabinoid metabolites are not commercially available. Hydroxylated and epoxygenated metabolites of anandamide (20-HETE-EA, 5,6-EET-EA, 8,9-EET-EA, 11,12-EET-EA, 14,15-EET-EA) and 14,15 epoxide of 2-AG (2-14,15-EG) can be purchased from Cayman Chemical.
5. Please note that at high substrate concentration (e.g., 100 μM) we observe enzyme inhibition. Therefore, a dose-response curve should always be performed. All quantitation experiments for kinetic purposes must be performed using substrate concentrations that are within the linear dynamic range of detection of the instrument used.
6. For chemical inhibition studies, a vehicle control should be included, and a negative control antibody (such as hen egg lysozyme) should be included in reactions containing inhibitory antibodies.
7. Cell seeding density, time of incubation, and endocannabinoid concentrations should be optimized depending on the cell type. Also note that the signal-to-noise ratios are significantly lower for whole-cell assays than for purified P450s and microsomes, due to the presence of other cellular lipid components in the organic extract.
8. Longer reaction times may be required when studying the reaction products of P450s with slower turnover rates, such as P450 2J2. This also holds true for microsomal studies.
9. If a precipitate persists after prolonged vortexing, additional solvent should be added to ensure complete solubilization prior to injection onto the column. DMSO may also be added up to 10 %, to improve solubility.
10. Since authentic standards for most P450-generated endocannabinoid metabolites are not currently available, structurally similar products may be used for generating standard curves that would allow to estimate metabolite concentrations.

Acknowledgements

This work was supported in part by the National Institutes of Health (Grants CA16954 and GM007767).

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