Journal of Steroid Biochemistry & Molecular Biology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

# A microsomal based method to detect aromatase activity in different brain regions of the rat using ultra performance liquid chromatography–mass spectrometry

Junyi Li, Patrick J. Oberly, Samuel M. Poloyac, Robert B. Gibbs*

*Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15261, USA*

ARTICLE INFO

*Article history:*
Received 2 November 2015
Received in revised form 22 February 2016
Accepted 20 April 2016
Available online xxx

*Keywords:*
Local estrogen production
Estradiol
Mass spectroscopy
CYP19A1

ABSTRACT

Aromatase (ARO) is a cytochrome P450 enzyme that accounts for local estrogen production in the brain. The goal of this study was to develop a microsomal based assay to sensitively and reliably detect the low levels of ARO activity in different brain regions. Enzyme activity was detected based on the conversion of testosterone to estradiol. Quantity of estradiol was measured using ultra performance liquid chromatography–mass spectrometry. Detection was linear over a range of 2.5–200 pg/ml estradiol, and was reproducible with intra- and inter-assay coefficients of variation (CV) <15%. Estradiol production using isolated microsomes was linear with time up to 30 min as well as linearly related to amount of microsome. Substrate concentration curves revealed enzymatic kinetics (hippocampus: $V_{max}$ and $K_m$: 0.57 pmol estradiol/h per mg microsome and 48.58 nM; amygdala: $V_{max}$ and $K_m$: 1.69 pmol estradiol/h per mg microsome and 48.4 nM; preoptic area: $V_{max}$ and $K_m$: 0.96 pmol estradiol/h per mg microsome and 44.31 nM) with testosterone used at a saturating concentration of 400 nM. Anastrozole treatment blocked ARO activity in hippocampal and ovarian microsomes, indicating that the assay is specific for ARO. Also, we showed that the distribution of the long form ARO mRNA (CYP19A1) in different regions of the brain is correlated with ARO activity, with highest levels in the amygdala, followed by preoptic area and hippocampus. In the frontal cortex, very little long form ARO mRNA, and little to no ARO activity, were detected. These findings demonstrate that the microsomal incubation (MIB) assay is a sensitive and reliable method for quantifying ARO activity in discrete brain regions.



## 1. Introduction

Many studies show that estrogens can have beneficial effects on brain function and cognition in rats and mice, as well as in non-human primates and humans [1–6]. In particular, estrogens have been shown to increase synaptic plasticity, modulate neurotransmitter activity, and enhance performance on a variety of cognitive tasks [7]. There also is evidence that estrogen replacement can reduce the risks of Alzheimer's related dementia in women, provided that therapy is initiated soon following either surgical or natural menopause [8,9]. Most of these studies have evaluated the effects of systemic estrogen treatments, assuming that brain levels of estradiol and estrone mirror levels in the systemic circulation. The brain, however, contains all of the enzymes necessary to synthesize estradiol locally, and recent studies suggest that local estrogen synthesis may have a far greater impact on neuronal function than systemic estrogen administration [10,11]

Aromatase (CYP19A1; ARO) is the Cytochrome P450 enzyme responsible for the production of estradiol from testosterone, and estrone from androstenedione [12]. Early studies focused on the importance of this enzyme in regulating sexually dimorphic, brain development and sex/mating behaviors in birds [13] as well as in rodents [14–17]. Recent studies show that brain ARO plays a critical role in regulating brain structures involved in the seasonal song development in song birds [18–20], as well as in the development of sexually dimorphic and sex-specific behaviors in rodents [21–24]. However, ARO is expressed in other regions of the brain as well. Evidence shows that different stress conditions as well as experimental disease models such as brain injury [25], experimental stroke [26], hypertension [27], and neuroinflammation [28] can induce ARO expression and activity in the hippocampus, cerebral cortex, and cerebellum in vitro and in vivo. Studies by Roselli [29,30], Garcia-Segura [31], and more recently by Takahashi [32] & Tabatadze [33] have demonstrated ARO

* Corresponding author.
*E-mail address:* gibbsr@pitt.edu (R.B. Gibbs).

http://dx.doi.org/10.1016/j.jsbmb.2016.04.013
0960-0760/

expression and activity in many brain regions, with highest levels in the amygdala, the bed nucleus of the stria terminalis (BNST) and preoptic area, and lower levels in hippocampus, cortex, and cerebellum. These regional differences suggest that local estradiol levels are determined both by systemic concentrations and local production.

The CYP19A1 gene locates on the long arm of chromosome 9. It spans 2863 bp. Segment 613–2124 bp is the coding region and contains 9 exons designated exons 2–10. There are two isoforms of the CYP19A1 gene in the brain. One is 430nt in length, and is associated with enzyme activity in the brain. The other is 300nt in length, is not associated with enzyme activity, and the function of which is unknown. Different brain regions have different combinations of the two gene types [34]. A recent study by Tabatadze et al. [33] evaluated the distribution of long form CYP19A1 mRNA and confirmed that it is similar to the pattern of enzyme activity described by others.

Several methods have been used to measure ARO activity. In clinical or in vivo studies, where substrate concentrations are not under strict control and in which formation rate cannot be measured directly, a metabolic ratio of 17beta-estradiol to testosterone is often used to indicate ARO activity. When studied in vitro, a more direct method of evaluating product formation is preferred, particularly when studied at $V_{max}$ where changes in metabolic ratio are not linearly related to substrate concentration. One commonly used method for measuring ARO activity in tissues is the tritiated water assay developed by Roselli and co-workers [35]. It has been used to detect ARO activity in ovarian, placental, avian brain and mammalian brain. The assay is relatively straightforward and simple to apply, however, it has limitations with respect to reproducibility and sensitivity. This poses challenges for measuring the very low levels of ARO activity in brain. The purpose of the current study was to develop a highly sensitive microsomal assay for measuring ARO activity and to show that this method can be used to measure activity in discrete regions of the brain in relation to expression of the long-form of CYP19A1 mRNA.

## 2. Method

35 Young female intact Sprague-Dawley rats were purchased from Hilltop Laboratories, Inc. Rats were individually housed for two weeks in our facility on a 12 h:12 h light/dark schedule with unrestricted access to food and water. On the day of dissection, animals were anesthetized using a mixture of 0.6 mg xylazine and 3 mg ketamine. Brains were removed and tissues from the hippocampus, frontal cortex, preoptic area, and amygdala were collected. Tissues were stored at −80 °C until use.

### 2.1. Microsomal incubation assay

#### 2.1.1. Microsomal extraction

Brain tissues were collected. Tissues from the same brain region (50–250 mg) were pooled, homogenized in 50 mM Tris buffer (pH 7.4) containing 150 mM KCl, 0.1 mM dithiothreitol (Fluka, USA), 1 mM EDTA and 20% glycerol (Fisher), and mixed with 0.113 mM butylated hydroxytoluene (BHT) and 0.100 mM phenylmethylsulfonylflouride (PMSF) at 4 °C. Samples were centrifuged at 20,000*g* for 33 min at 4 °C. The supernatant was collected and centrifuged at 140,000*g* to obtain a microsomal pellet. Microsomal pellets were then dissolved in 200 μl 0.02 M Tris buffer containing 0.25 M sucrose. For this experiment, hippocampi were pooled from two rats, amygdala from three rats, and preoptic area from three rats to generate one data point for each brain region. Protein levels were determined by Bio-rad protein assay (Bio-Rad Laboratories, Inc.). Microsomes were stored at −80 °C until used.

#### 2.1.2. Microsomal incubation assay

To measure ARO activity, 100–200 μg microsomes prepared from different brain regions were incubated with testosterone, and the amount of estradiol produced was measured by LC–MS/MS. Microsomes were added to the microsomal incubation buffer, which is a sodium phosphate buffer pH 7.4 containing 5.0 mM $MgCl_2$. Testosterone was added to each sample tube to a final concentration of 400 nM. To start the reaction, 50 μl of 0.02 M Nicotinamide adenine dinucleotide phosphate (NADPH) was added to each tube, vortexed for 5 s and then placed at 37 °C. After 30 min, the reaction was stopped by rapid cooling on wet ice. The total volume of the reaction was 1.0 ml.

#### 2.1.3. Estradiol extraction and derivatization

Estradiol was collected from the sample by liquid-liquid extraction using *n*-Butyl chloride and then derivatized with dansyl chloride. Samples were first spiked with internal standard 25 μl 2,4,16,16,17-d5-17 beta-estradiol (1 ng/ml in methanol). 3 ml *n*-Butyl chloride was then added and vortexed for 1 min. The tubes were then centrifuged at 4770*g* at room temperature (RT) for 10 min and the organic layer was transferred to salinized culture tubes and dried down under a soft steam of nitrogen at 37 °C for 20 min. Residues were derivatized in 0.1 ml buffered dansyl chloride solution (a 1:1 mix of acetonitrile: water, pH 10.5), heated at 60 °C for 3 min, and then transferred to glass vials for LC–MS/MS analysis.

#### 2.1.4. Estradiol detection

We used a modified version of a UPLC–MS/MS method described by Nelson et al. [36]. Liquid chromatography was performed using an Acquity ultra performance LC autosampler (Waters, Milford, MA). Analytes were separated on a UPLC BEH C-18, 1.7 μm (2.1 × 150 mm) reverse-phase column (Waters, Milford, MA). Column temperature was maintained at 55 °C. Mobile phases, delivered at a flow rate of 0.3 ml/min, consisted of (A) acetonitrile and (B) 0.1% formic acid in water, at an initial mixture of 50:50 A and B. Mobile phase B was maintained at 50% for 1 min and then increased to 85% in a linear gradient over 3 min, where it remained for 1 min. This was followed by a linear return to initial conditions over 1.5 min. Total run time per sample was 6.5 min and all injection volumes were 7.5 μl.

Mass spectrometric analysis of analyte formation was performed using a TSQ Quantum Ultra (Thermo Fisher Scientific, San Jose, CA) triple quadrupole mass spectrometer coupled with heated electrospray ionization source (HESI) operated in negative selective reaction monitoring (SRM) mode with unit resolutions at both Q1 and Q3 set at 0.70 full width at half maximum. Quantification by SRM analysis of estradiol was performed by monitoring the *m*/*z* transitions. The retention time for estradiol was 5.64 min for native estradiol and 5.62 min for the deuterated internal standard. Parameters were optimized to obtain the highest $[M-H]^-$ ion abundance and were as follows: capillary temperature, 350 °C, vaporizer temperature, 355 °C, spray voltage, 4000 kV, and a source collision-induced dissociation set at 0 V. Sheath gas and auxiliary gas were set at 20 and 34 respectively. Scan time was set at 0.01 s and collision gas pressure was set at 1.5 mTorr. Analytical data was acquired and analyzed using Xcalibur software version 2.0.6 (Thermo Finnigan, San Jose, CA).

#### 2.1.5. Aromatase activity calculation

Calibration curves were prepared and extracted along with unknowns. Ideally calibration curves would be prepared in a matrix of inactivated microsomes; however, repeated preparation of large amounts of microsomes from brain is costly in both time and rats. We therefore tested the potential of 2-hydroxypropyl-β-cyclodextrin (HPCD) (Alorich, USA) to be used as an alternative

Please cite this article in press as: J. Li, et al., A microsomal based method to detect aromatase activity in different brain regions of the rat using ultra performance liquid chromatography–mass spectrometry, J. Steroid Biochem. Mol. Biol. (2016), http://dx.doi.org/10.1016/j.jsbmb.2016.04.013