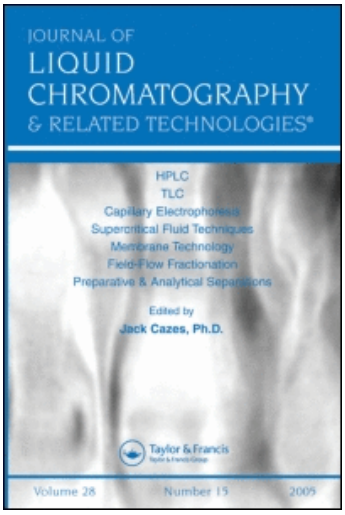


## Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# Rapid Assay of Hypothalamic Aromatase Using High Performance Liquid Chromatography

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**To cite this Article** Wilke, D. L. and Braselton Jr., W. E.(1983) 'Rapid Assay of Hypothalamic Aromatase Using High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 6: 5, 861 — 870

**To link to this Article: DOI:** 10.1080/01483918308067008

**URL:** http://dx.doi.org/10.1080/01483918308067008

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(5), 861-870 (1983)

# RAPID ASSAY OF HYPOTHALAMIC AROMATASE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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## ABSTRACT

A rapid assay for hypothalamic aromatase has been developed using HPLC. Each assay tube contained rat anterior hypothalamus homogenized in Na phosphate buffer (pH 7.0), NADPH regenerating system, unlabelled estradiol and estrone, and 10 μCi of $^{3}$H-androstenedione. After agitation at 37°C for 3 hours, the reaction mixture was extracted with $CH_2Cl_2$, partitioned between 90% methanol and hexane, and the methanol layer chromatographed on a Waters 10μ C-18 Radial-PAK column using acetonitrile/water (70:30). The estrogens were collected together and rechromatographed with THF/water (35:65). The estrone peak was collected, and the $^{3}$H-estrone produced was determined by liquid scintillation. The estrone collected from the second chromatographic step was found to be at least 95% pure by methylation and rechromatography. Using the HPLC procedure, enzymatic $^{3}$H-estrone formation was found to be linear in a time and protein dependant manner.

## INTRODUCTION

Aromatization of androgens to estrogens in the hypothalamus is a critical step in sexual differentiation of the mammalian



 0148-3919/83/0605-0861$3.50/0

brain and the regulation of mammalian reproductive function (1-3). Although aromatizing activity is central to the normal control of reproduction, hypothalamic aromatase is active at very low levels and radioactive tracer methods are necessary for its detection. In tissues with high aromatase activity, such as placenta and developing brain, assays relying on production of $^{3}H_2O$ from tritiated substrate have been successful, but the results obtained with this assay do not agree quantitatively with assays which detect the production of tritiated estrogens (4). Previous methods for detection of aromatization in the adult hypothalamus have relied on separation of the radiolabelled estrogens by solvent partition, thin layer chromatography, derivatization, and reverse isotope dilution and recrystallization to constant specific activity (1,2,5). Methods involving recrystallization are time consuming and recoveries must be calculated using dual isotope techniques. The method which we report here relies on two successive HPLC steps to isolate the radiolabelled estrogens produced, and allows calculation of recovery using U.V. absorbance of reference steroids added as carriers.



## MATERIALS AND METHODS

### Substrate Purification

Two successive HPLC procedures were necessary to insure substrate purity and to remove all traces of androsta-1,4,6-triene-3,17-dione, a potent inhibitor of the aromatase enzyme system. (1,2,6,7)-$^{3}$H-Androst-4-ene-3,17-dione was obtained from New

England Nuclear (Boston MA) and purified by reverse phase chromatography using acetonitrile/water (40:60) on an Altex Ultrasphere ODS column. The androstenedione peak was collected, and rechromatographed on the same column using THF/water (35:65) as the eluent.

Hypothalamic Incubation and Sample Preparation

Adult male Sprague-Dawley rats were killed by decapitation, and the entire brain removed and frozen on dry ice. Anterior hypothalamus containing the median pre-optic area was removed using a dissection similar to that reported by Naftolin (6). The hypothalamus was homogenized in 0.05 M phosphate buffer (pH 7.0), and microgram quantities of estradiol and estrone were added to allow easy calculation of recovery following incubation. An NADPH regenerating system was added, and the reaction was started by the addition of 10 μCi of (1,2,6,7)-$^{3}$H-androst-4-ene-3,17-dione (90 Ci/mmole), purified as described. Each assay tube contained 10 hypothalami, 200nM $^{3}$H-androstenedione, 2.4 μM NADPH, 12 μM glucose-6-phosphate, and 0.4 Units of glucose-6-phosphate dehydrogenase in a final volume of 0.5 ml. Assay tubes were incubated with agitation for 3 hours at 37°C. Reactions were terminated by the addition of 5 ml of methylene chloride. Blanks were prepared by addition of methylene chloride at zero time, or by incubation of boiled cortex homogenates for 3 hours.

Each incubation tube was extracted twice with 5 ml of methylene chloride, the combined extracts dryed over anhydrous $Na_2SO_4$, and evaporated to dryness under $N_2$. Each sample

was dissolved in 0.5 ml of methanol/water (90:10), and extracted with 1.0 ml of hexane to remove non-polar lipids. The methanol/water layer containing the estrogens was evaporated to dryness and redissolved in 50 µl of methanol prior to chromatography.

Placental Microsome Incubation

Microsomes were prepared from a healthy full-term human placenta as described by Ryan (7), and stored in sucrose phosphate buffer at -70°C until used. Placental microsomes were incubated in 0.05M phosphate buffer as described above for hypothalamic homogenates. Placental microsomes served as positive controls whenever hypothalamic homogenates were incubated.



High Performance Liquid Chromatography

Two HPLC systems were developed for the assay, and two additional systems used to confirm the purity of the peaks collected during the assay (Figure 1):

**System A**: acetonitrile/water (70:30), 1.0 ml/min, Waters 10µ radial-PAK reverse phase (C-18) cartridge;

**System B**: THF/water (35:65), 2.5 ml/min, Waters 10µ radial-PAK reverse phase (C-18) cartridge;

**System C**: acetonitrile/THF/water (55:5:40), 1.0 ml/min, Altex 5µ Ultrasphere ODS column (15 cm length);

**System D**: cyclohexane/n-octanol (95:5), 2.0 ml/min, Waters 10µ radial-PAK normal phase cartridge.

The HPLC system consisted of a Waters model M6000A pump (Milford MA) with a Model 440 U.V. detector at 254 nm (systems A,B) or 280

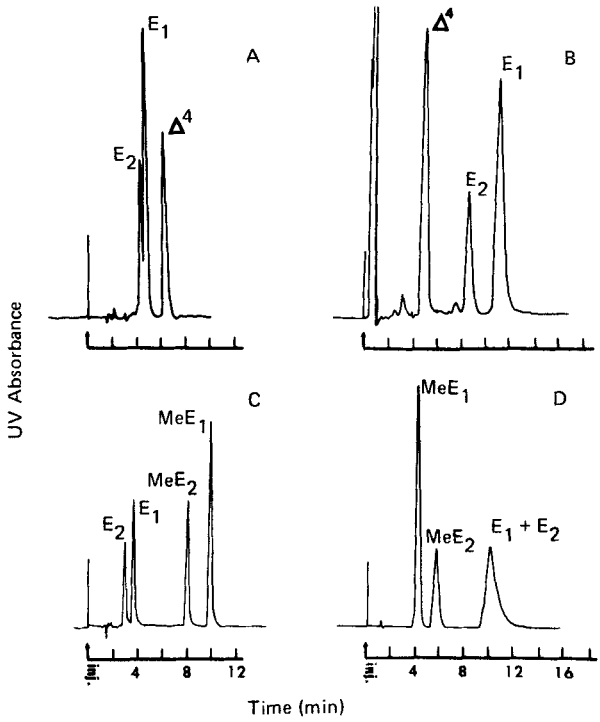


FIGURE 1. Chromatograms of reference steroids. For chromatographic conditions, see text. $E_1$ = estrone, $E_2$ = 17B-estradiol, $\Delta^4$ = androstenedione, $MeE_1$ = estrone 3-methyl ether, $MeE_2$ = 17B-estradiol 3-methyl ether.

nm (systems C,D). Steroids were quantified by a Waters 760 Data Module recording integrator.

Following sample preparation as described above, the samples were chromatographed on system A, and the estrone and estradiol collected together for rechromatography. The estrone peak was collected during chromatography on system B, and the $^3$H-estrone present was quantified by liquid scintillation in a

Packard Tri-Carb, (Model 460 C). Recovery of estrone was calculated from U.V. absorbance during chromatography on system B.

Method Validation

In order to verify the purity of the estrone peaks collected and counted following chromatography on system B, several test incubations were performed. The first incubation was carried out in 6 tubes, each containing a different amount of placental microsomes. After incubation as described above, the estrogenic products in each tube were subjected to chromatography using system A, then split into two aliquots. The first aliquot was chromatographed on system B, and the specific activity of the estrone collected was determined. The second aliquot was run on system B, the estrone peak collected, and then methylated by the method of Brown (8). The 3-methyl ether of estrone was then chromatographed on system C, and the specific activity of the estrone methyl ether compared against the specific activity of the estrone obtained during chromatography on system B.

Peak purity was also checked for incubations containing homogenates of hypothalamic tissue. Three tubes, each with 10 hypothalami, were incubated as described above. Peak purity of the estrone produced following chromatography on system B was determined as described above for placental microsomes, however following methylation the specific activity of the estrone methyl ether produced was determined on both system C and system D.

## RESULTS AND DISCUSSION

Chromatography with system A was useful in separating the estrone produced from the highly radioactive $^{3}$H-androstenedione

substrate. Since the estrogens eluted first on system A, the chance of the androstenedione "tailing into" the estrogen peaks was eliminated. The second chromatographic step with system B, then separated the estrogens from each other, and from any residual androstenedione which might remain. Calculation of estrone recovery following system B showed recoveries which averaged 70 - 80 % of the estrone added to the incubation tubes. Figure 2 shows a radiochromatogram (HPLC system B) of the estrone produced by incubation of $^{3}H$-androstenedione with hypothalamic tissue in comparison with that seen in a blank incubation. Since



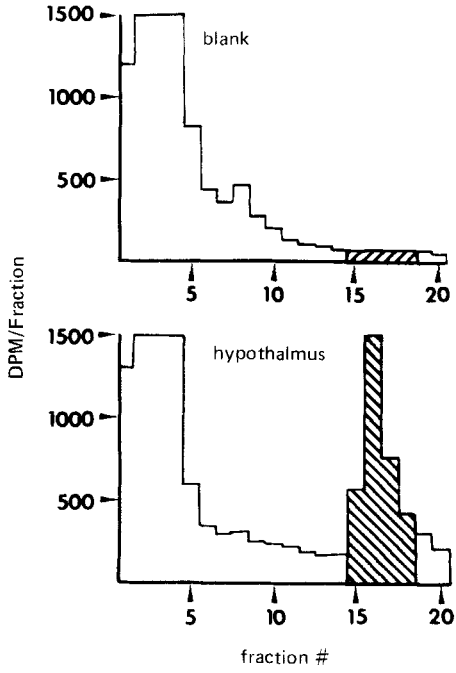


FIGURE 2. Radiochromatogram of fractions collected from system B chromatography of zero-time blank and hypothalamic homogenate incubation. Fractions collected every 30 seconds beginning at 4 minutes.

hypothalamic tissue has been shown to contain little 17B-dehydrogenase (4), and since only trace amounts of estradiol were observed following incubation of hypothalamic homogenates (Fig 2), the estradiol peak was not further characterized, and was not used in the calculation of hypothalamic aromatase activity.

An average purity of 95% or greater (Table 1) was shown by collection of estrone following chromatography on system A, chromatography on system B, methylation, and rechromatography on system C or D. Since the criterion for purity using recrystallization is normally a 5% or smaller change in isotope ratio, the purity of estrone produced by this method is at least as good as that produced by repeated recrystallization.



TABLE 1.
Purity of Estrone Produced by Aromatization Following Reverse Phase Chromatography with 35% THF.

| sample # | spec. activity after THF dpm/µg estrone | spec. activity following methylation reverse phase dpm/µg* | %THF | normal phase dpm/µg* | %THF |
|---|---|---|---|---|---|
| Hypothalamic homogenate | | | | | |
| 1 | 213 | 224 | 105.% | 221. | 103.% |
| 2 | 452 | 411 | 95.6% | 371 | 86.3% |
| 3 | 266 | 242 | 95.6% | 247 | 97.6% |
| | | | 98.7±3.1 | | 95.6+5 |
| Placental microsomes | | | | | |
| 1 | 119 | 118 | 99.2% | | |
| 2 | 1050 | 1090 | 104. % | | |
| 3 | 793 | 752 | 94.8% | | |
| 4 | 191 | 174 | 91.1% | | |
| 5 | 686 | 684 | 99.7% | | |
| 6 | 402 | 363 | 90.3% | | |
| | | | 96.5±2.4 | | |

* calculated on the basis of µg of original estrone added.

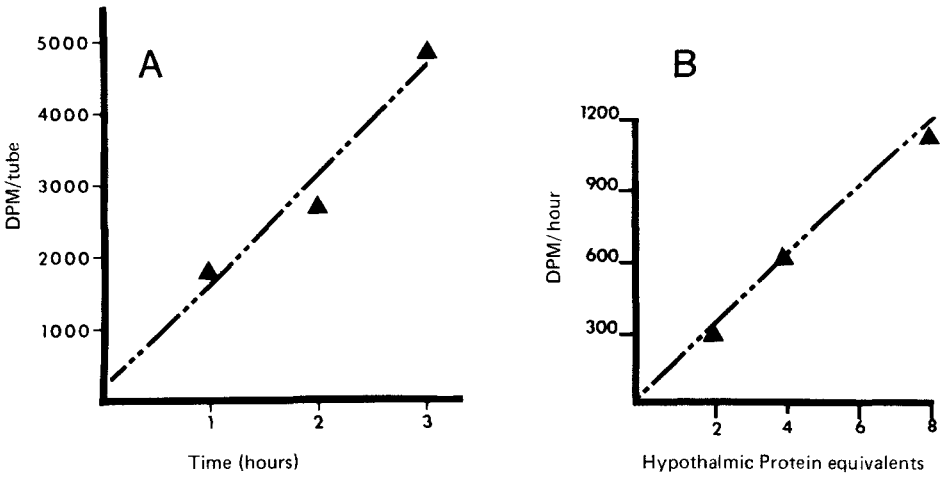


FIGURE 3. Time (A) and protein (B) dependancy of incubation of hypothalamic homogenates. Incubation conditions described in text.



Both time and protein dependency were determined using placental microsomes (data not shown), and confirmed using hypothalamic incubations. This incubation technique showed linear time dependancy to 3 hours and was protein dependant with hypothalamic protein equivalent to 2 to 10 hypothalami (Figure 3).

The results indicate that hypothalamic aromatase may be measured following separation of estrogens on HPLC system A and isolation and quantification of estrone on HPLC system B. The method offers good recoveries of 70 - 80%, is rapid and easily performed, produces products with a high degree of purity, and is sensitive, allowing the detection of less than 10 femtomoles of tritiated estrogen produced by aromatization.

ACKOWLEDGEMENT

This work was funded in part by the Michigan State University Center for Environmental Toxicology, E. Lansing, Michigan.

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