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A COMPARISON OF METHODS MEASURING AROMATASE ACTIVITY IN HUMAN PLACENTA AND RAT OVARY

H.S. PURBA* and A.S. BHATNAGAR

Research Department, Pharmaceutical Division, CIBA-GEIGY Limited, CH-4002 Basel, Switzerland

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The single step chromatographic product isolation method using [4-^{14}C]-androstenedione and the tritiated water method using either [1β,2β-^{3}H]- or [1β-^{3}H]-androstenedione have been used to determine a suitable method to measure the aromatase activity in rat ovarian 1000 g supernatant and human placental microsomes.

The single step product isolation method using [4-^{14}C]-A_4 reveals the presence of four distinct [4-^{14}C]-labelled products in the rat ovary of which only the synthesis of estradiol is markedly inhibited by CGS 16949A, a well established aromatase inhibitor. In the human placenta, the formation of both [4-^{14}C]-estrone and [4-^{14}C]-estradiol is strongly inhibited by CGS 6949A. Therefore, in the rat ovary spurious results are obtained if accumulative radiolabelled product formation is measured without characterisation of the products.

The V_{max} in the rat ovary using [1β,2β-^{3}H]-A_4 as a substrate is 13.7 pmol/h/mg compared to 2.9 pmol/h/mg when [1β-^{3}H]-A_4 is used. In the human placenta, the V_{max} is similar using either [1β,2β-^{3}H]-A_4 or [1β-^{3}H]-A_4 (1.21 and 1.27 nmol/h/mg, respectively). Consistent results are obtained for the human placenta using either the single step chromatographic product isolation method or the tritiated water method. However, in the rat ovary the more suitable method of the two used to measure the aromatase activity is the tritiated water method employing [1β-^{3}H]-A_4 as a substrate.

Aromatase activity in the rat ovary during estrus cycle was measured using the tritiated water method employing [1β-^{3}H]-A_4 as a substrate. A peak of aromatase activity at proestrus was seen which returned rapidly to its basal level at estrus. Plasma estradiol concentrations were in parallel with the aromatase activity.

KEY WORDS: Aromatase, human placenta, rat ovary, methods.

INTRODUCTION

The conversion of androgens to estrogens is carried out by the enzyme aromatase, primarily in the ovary in females of reproductive maturity. The human placenta, however, has served as the source of aromatase for its characterisation[1–3] due to the abundance of aromatase in this tissue. Although the human placenta has also been the source of aromatase for a variety of *in vitro* inhibition studies used to characterise novel drugs for the treatment of estrogen-dependent cancers, *in vivo* profiling of these inhibitors has been done predominantly in rats.[4–6] Thus, this study was undertaken to characterise the aromatase assay in rat using the human placenta as a reference tissue.

The aromatase reaction utilizes three moles each of NADPH and oxygen per mole

* Correspondence to: Dr. H.S. Purba, c/o CIBA-GEIGY Limited, K-125.8.20, CH-4002 Basel, Switzerland.

of androstenedione (C-19 steroid) converted to estrogen.[7] The first two hydroxylations occur at the 19-methyl group of the androgen but there is considerable speculation about the third step involved in the aromatisation. Recent work by Simpson *et al.*[8] has shown that a single protein expressed as a result of transfection of COS1 cells with a vector containing the P-450$_{AROM}$ cDNA, is capable of catalysing all three steps involved in the aromatisation reaction.

In previous studies, the product isolation method using [4-^{14}C]-androstenedione or the tritiated water method using [1β,2β-^{3}H]- or [1β-^{3}H]-androstenedione as a substrate have been used to measure the aromatase activity in various tissues. Product isolation has either been carried out very simply through single step thin layer chromatography[5] or by a combination of several separation steps.[9]

In preliminary studies,[10] it was not possible to measure aromatase accurately in the rat ovary using a single step chromatographic product isolation method because of the appearance of unidentified metabolites of androstenedione, one of which co-chromatographed with estrone and whose formation was not inhibited by an established aromatase inhibitor. Therefore it was decided to perform a systematic study to define precise assay conditions for the measurement of aromatase activity in the rat ovary during the estrus cycle and human placenta. The enzyme kinetics of aromatase were also examined in these tissues in the absence and presence of a specific aromatase inhibitor CGS 16949A (4-(5,6,7,8-tetrahydroimidazo[1,5-a]pyridin-5-yl) benzonitrile hydrochloride) to validate further different assays which used three separate radiolabelled forms of androstenedione. CGS 16949A, which has previously been shown to be a very potent and highly specific inhibitor of aromatase in the human placenta *in vitro*[5] was used as a reference drug to validate the assays.

MATERIALS

Chemicals

[4-^{14}C]-Androstenedione ([4-^{14}C]-A$_4$), [4-^{14}C]-estradiol ([4-^{14}C]-E$_2$), [4-^{14}C]-estrone ([4-^{14}C]-E$_1$; 50–60 mCi/mmol), [1β-^{3}H]-A$_4$ (27.4 Ci/mmol) and [1β,2β-^{3}H]-A$_4$ (40–60 Ci/mmol) were purchased from New England Nuclear (Dreieich, F.R.G.). Unlabelled androstenedione was purchased from Steraloids Inc. (Wilton, U.S.A.). CGS 16949A was synthesised at Ciba-Geigy Limited. Estradiol radioimmunoassay kit was purchased from Baxter GmbH (Düdingen, Switzerland). All biochemicals were obtained from Sigma Chemical Company. Solvents and miscellaneous chemicals were from E. Merck and were of analytical grade.

Animals

Adult female Sprague-Dawley rats (190–250 g) were used. Estrus cycles were determined by vaginal cytology.

METHODS

Preparation of tissues

Ovaries were removed from cyclic rats at 9.00 h on different days of the 4-day estrus

cycle, weighed and stored at −20°C until preparation of supernatants. Phosphate buffer (4 ml; 0.05 M; pH 7.4) was added to a pair of ovaries from each animal. The ovaries were subjected to 3 or 4 quick bursts of Kinematica Polytron followed by 4 slow bursts (1,000 r.p.m.) of teflon homogeniser. The homogenate was then centrifuged at 1,000 g for 20 min at 4°C. The resulting supernatant was decanted into a clean vessel and 0.5 ml aliquots were made from a stirring suspension at low speed into clear plastic tubes, snap frozen and stored at −20°C.

The microsomal fraction was prepared from freshly delivered human term placentae. The tissue was washed in ice cold 1.15% KCl and freed of membranes and blood vessels. The tissue was placed in 0.25 M sucrose (1.5 ml/g tissue) and cut into small pieces with surgical scissors. The tissue was homogenised in an Osterizer blender using 4 or 5 30 s bursts at low speed with 15 s cooling periods. Portions (50 ml) of the homogenate were subjected to Kinematica Polytron using 4 or 5 15 s bursts at speed 5 with 10 s cooling periods. The homogenate was centrifuged at 20,000 g for 30 min (Sorvall RC-5 Centrifuge, Du Pont Instruments). The clear supernatant was centrifuged at 148,000 g for one hour (Centrikon 2070 Centrifuge). The pellets were washed in phosphate buffer (0.05 M; pH 7.4) using a teflon homogeniser. The washed pellets were pooled together and made up to about 30 ml with phosphate buffer and centrifuged at 100,000 g for one hour. The resulting pellet was washed again and recentrifuged at 100,000 g for one hour. The final pellet was resuspended in 32 ml phosphate buffer using teflon homogeniser and 100 μl aliquots were made from a stirring suspension into clear plastic tubes, snap frozen and stored at −20°C.

Protein content in both tissues was determined by the method of Lowry *et al.*,[11] using a Shimadzu UV-spectrophotometer.

Aromatase activity using [4-^{14}C]-A_4 as a substrate (single step chromatographic product isolation method)

Incubations were performed in glass-stoppered 15-ml centrifuge tubes at 37°C containing [4-^{14}C]-A_4 (400 nM), excess NADPH (0.24 mM), protein (0.05–0.1 mg) and phosphate buffer (0.05 M; pH 7.4). The final volume of the incubation mixture was 1.0 ml. Control incubations were also performed without any enzyme. The reaction was started with the addition of protein and stopped after 20 min with the addition of diethyl-ether (7 ml). Following extraction, the tubes were centrifuged at 2,000 g for 5 min in a bench top centrifuge. The organic layer was transferred into a clean set of tubes after freezing the aqueous layer in dry ice. The organic layer was evaporated under a stream of nitrogen and reconstituted in 70 μl acetone. The reconstitute was spotted on a TLC plate (Kieselgel 60 F-254, E. Merck) using a Camag Linomat IV spotter. In addition, [4-^{14}C]-A_4, [4-^{14}C]-E_2 and [4-^{14}C]-E_1 were spotted on three different tracks as reference standards. The plate was developed in a TLC tank containing chloroform-methanol (95:5). Radioactivity was measured on a Berthold TLC Digital Autoradiograph linked to a Compaq 386/20 computer and a Hewlett Packard printer. Radioactive spots corresponding to [4-^{14}C]-A_4, [4-^{14}C]-E_2 and [4-^{14}C]-E_1 were integrated. Aromatase activity was calculated as a sum of counts corresponding to estrone and estradiol. The intra-assay variation of total estrogens produced at a substrate concentration of 50 nM was 13.3% and 6.8% ($n = 5$) for the rat ovarian tissue and human placenta, respectively. The corresponding inter-assay variation values were 18.2% and 7.0%.

Aromatase activity using [1β-^{3}H]-A_4 or [1β,2β-^{3}H]-A_4 as a substrate (tritiated water method)

Incubation conditions were the same as above except that tritiated androstenedione was used and the reaction was stopped with the addition of 10 ml dichloromethane. Control incubations were also performed without any enzyme. Following extraction, the tubes were centrifuged at 2,000 g for 10 min. 0.5 ml of the aqueous phase was removed and placed in a glass tube to which 1.0 ml of 5% charcoal suspension was added and left to stand for at least 15 min at room temperature. The tubes were then centrifuged at 2,500 g for 20 min. Two 0.5 ml aliquots were removed, added to scintillation vials containing 10 ml Irgascint A300 (Ciba-Geigy) and counted in a liquid scintillation counter (Kontron) using nitromethane as a quenching standard. Counts from the control incubation were subtracted from each incubation counts. The intra-assay and inter-assay variation of the tritiated water released from either 1β-^{3}H]-A_4 or [1β,2β-^{3}H]-A_4 (50 nM) in the rat ovarian tissue and human placenta was less than 5%.

Effect of CGS 16949A on aromatase activity in rat ovarian tissue and human placental microsomes

Initial velocity conditions were established employing low substrate concentrations for linear production of estrogens. Incubation conditions were similar to those described above. In the control set, incubations contained varying concentrations of either [4-^{14}C]-A_4, [1β-^{3}H]-A_4 or [1β,2β-^{3}H]-A_4. In another set of incubations, CGS 16949A (10 nM) was added in addition to the presence of either of the three forms of radiolabelled androstenedione. Assay procedures were similar to those described above. Kinetic parameters were derived from a Lineweaver-Burk plot using a linear regression programme.

Aromatase activity during estrus cycle

Groups of rats exhibiting at least three regular cycles as assessed by daily vaginal smears were sacrificed at 9.00 h and 1,000 g ovarian supernatants were prepared from each animal from each stage of the estrus cycle. Aromatase activity was measured using [1β-^{3}H]-A_4 as a substrate (380 nM). The reaction time was 30 min. Plasma estradiol concentrations were also measured using a specific radioimmunoassay.

RESULTS

Kinetics of aromatase using differently labelled androstenedione as a substrate

Using our product isolation method with [4-^{14}C]-A_4 as a substrate, the K_m and the V_{max} of aromatase in the human placenta was 50.1 nM and 3.16 nmol/h/mg, respectively (Table I). However, in the rat ovary, the use of this product isolation method yields a higher K_m value of 118.2 nM and a V_{max} of 430 pmol/h/mg. The measurement of kinetic parameters in the human placenta using the tritiated water method employing either [1β,2β-^{3}H]-A_4 or [1β-^{3}H]-A_4 as a substrate produced K_m values of 50.4 and 52.3 nM, respectively. The corresponding V_{max} values were 1.21 and 1.27 nmol/h/mg. In the rat ovary, however, the two methods gave widely differing results. Using either

TABLE I
Aromatase kinetic parameters

Substrate	[4-^{14}C]-A_4	[1β,2β-^{3}H]-A_4	[1β-^{3}H]-A_4
Human placenta			
K_m (nM)	50.1[a]	50.4	52.3
V_{max} (nmol/h/mg)	3.16	1.21	1.27
Rat ovary			
K_m (nM)	118.2	234.5	40.8
V_{max} (pmol/h/mg)	430.2	13.7	2.9

[a] Each value is an average of two determinations.

[1β,2β-^{3}H]-A_4 or [1β-^{3}H]-A_4 as a substrate, the K_m values were 234.5 and 40.8 nM, respectively. The corresponding V_{max} values were 13.7 and 2.9 pmol/h/mg.

Effect of CGS 16949A on aromatase activity

Using [4-^{14}C]-A_4 as a substrate, the percentage inhibition is more pronounced in the human placenta compared to the rat ovary. In the human placenta, other than substrate, only two spots are seen corresponding to estrone and estradiol (Figure 1). The appearance of both these spots is strongly inhibited by CGS 16949A (Figure 2). The rat ovary, however, produces four distinct ^{14}C-labelled products, two of which have chromatographic mobilities corresponding to estradiol and estrone and two unidentified metabolites, M1 and M2 (Figure 1). However, CGS 16949A is able to inhibit significantly the formation of estradiol only (Figure 2). Thus the spot with the same R_f value coresponding to estrone must contain a large proportion of a product

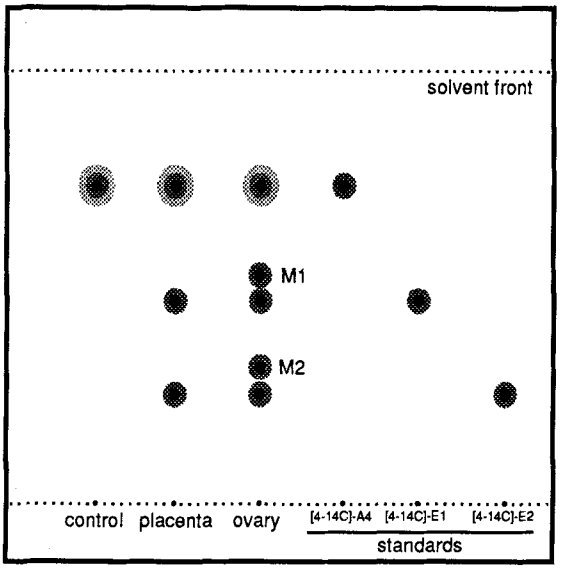

FIGURE 1 Diagrammatic representation of the TLC radiograph. M1 and M2 are the unidentified metabolites arising form the rat ovary.

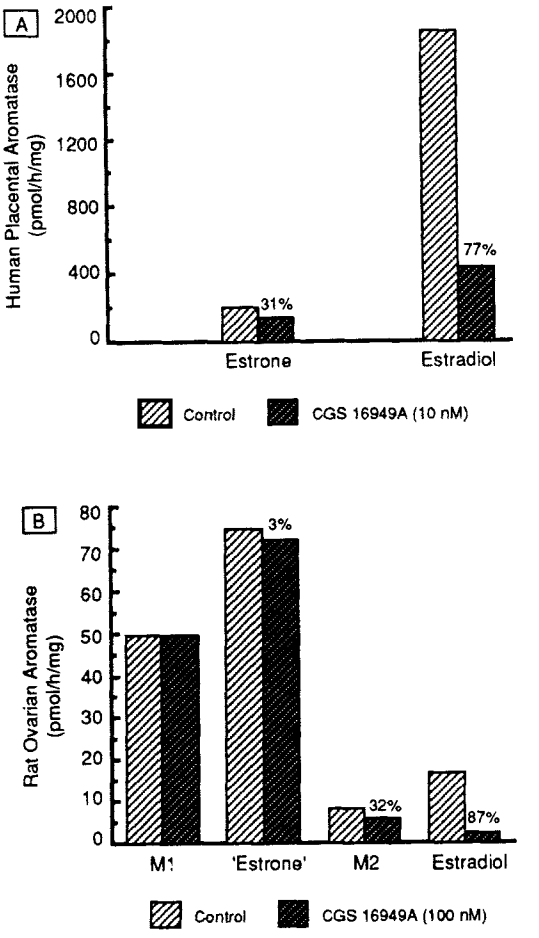

FIGURE 2 Effect of CGS 16949A (% inhibition) on individual product formation in (A) human placenta and (B) proestrus rat ovary using [4-^{14}C]-A_4 as a substrate.

which is not an estrogen. Therefore, when total estrogen (spots corresponding to estradiol and estrone) is quantitated by this method, the rat ovarian aromatase kinetic parameters obtained differ significantly (Table I; Figure 3) from the human placental parameters. K_i values derived for CGS 16949A using three radiolabelled substrates in human placenta and rat ovary are shown in Table II. The K_i values for CGS 16949A in human placenta are in the same range using both methods. From Table II it is evident that only the use of [1β-^{3}H]-A_4 in the rat ovary gives results comparable to those with human placenta. Using [1β,2β-^{3}H]-A_4 as a substrate in the tritiated water method, no kinetic parameters of inhibition can be determined for CGS 16949A because of its lack of inhibition at higher substrate concentrations.

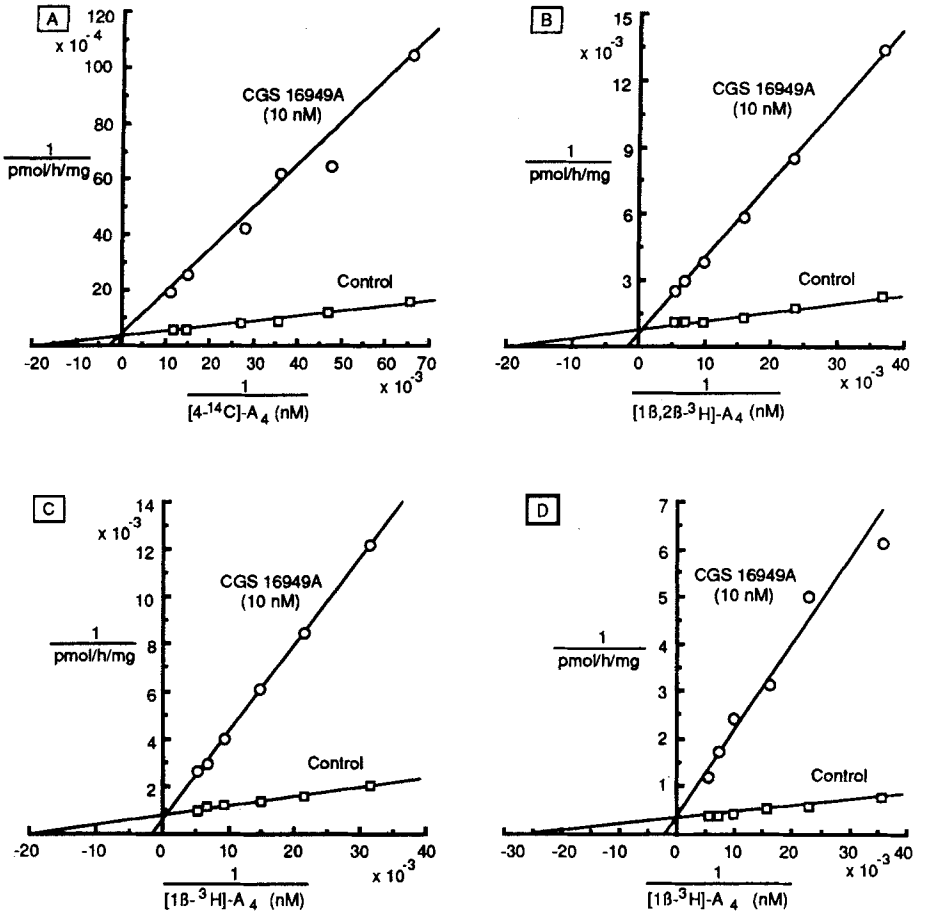

FIGURE 3 Typical Lineweaver-Burk plots showing the effect of CGS 16949A on (A) aromatase activity in the human placenta using [4-^{14}C]-A_4, (B) using [1β,2β-^{3}H]-A_4, (C) using [1β-^{3}H]-A_4 as a substrate and (D) on aromatase activity in the rat ovary using [1β-^{3}H]-A_4 as a substrate.

TABLE II
Inhibition of aromatase by CGS 16949A

Substrate	K_i (nM)		
	[4-^{14}C]-A_4	[1β,2β-^{3}H]-A_4	[1β-^{3}H]-A_4
Human placenta	1.16[a]	0.83	0.79
Rat ovary	324.1	Could not be measured	0.50

[a] Each value is an average of two determinations.

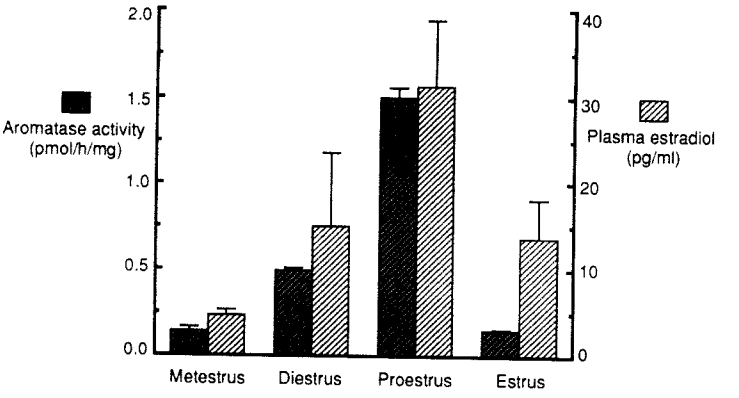

FIGURE 4 Plasma estradiol concentrations and the aromatase activity during estrus cycle using [1β-^{3}H]-A_4 as a substrate (mean $\pm$ s.e.m., $n = 5$).

Aromatase activity during estrus cycle

Figure 4 shows the aromatase activity during the estrus cycle as assessed by the tritiated water released from [1β-^{3}H]-A_4. Maximum aromatase activity occurs at proestrus which returns to its basal level at estrus and is strongly inhibited by CGS 16949A at all stages of the cycle (data not shown). Plasma estradiol concentrations are found to parallel the aromatase activity.

DISCUSSION

Aromatase kinetic parameters in the human placenta are similar using either the single step chromatographic product isolation method or the tritiated water method. On the other hand, in the rat ovary, aromatase kinetic parameters differ widely depending on the method used. The single step product isolation method using [4-^{14}C]-A_4 as a substrate produces a higher K_m of 118.2 nM because of an overestimation of estrogenic (or non-estrogenic) products. The tritiated water method using [1β,2β-^{3}H]-A_4 as a substrate also produces inaccurate results because of the relatively higher apparent V_{max} and the lack of inhibition by CGS 16949A, an established inhibitor of aromatase, especially at higher substrate concentrations. In the rat ovary, using the 1,000 g supernatant, the only comparable aromatase kinetic parameters to those obtained in the human placental microsomes are achieved by using the tritiated water method employing [1β-^{3}H]-A_4 as a substrate. As one of the aims of this study was to measure ovarian aromatase activity in animals individually and to correlate this with circulating estradiol concentrations during estrus cycle, and since microsomal yield of enzyme was not high enough in individual ovaries, 1,000 g supernatant was used.

The product isolation method using [4-^{14}C]-A_4 reveals the existence of four ^{14}C-labelled products in the rat ovary. Of these four products, CGS 16949A is able to inhibit significantly the synthesis of estradiol only. Therefore, the lack of inhibition of 'estrone' production suggests that this ^{14}C-labelled product co-chromatographing with authentic [4-^{14}C]-E_1 is probably not estrone but another hitherto unidentified

metabolite of androstenedione; the other two metabolites, M1 and M2 also remain unidentified. In the human placenta, [4-^{14}C]-A_4 yields estrone and estradiol only and their production is strongly inhibited by CGS 16949A even at a low concentration (K_i = 1.16 nM). The unidentified metabolites arising from the rat ovary and not from the human placenta suggests that they are not the common intermediate products of aromatisation but rather metabolites of another enzyme(s) in the rat ovary. Although there is now growing support for the existence of 19-hydroxy-androstenedione and 19-oxo-androstenedione as products of aromatisation in their own right rather than free obligatory intermediates, both in the rat ovary[12] and in the human placenta,[13] we did not detect these products in the human placenta using our product isolation technique. Therefore, the unidentified metabolites M1 and M2 in the rat ovary are probably not 19-hydroxy- or 19-oxo-androstenedione.

Before the advent of autoradiographic scanners, the standard procedure for the isolation of products on a TLC plate was localising the radioactive product co-chromatographing with a cold standard under u.v. light or spraying with a colour reagent and then scraping the localised area followed by liquid scintillation counting. The use of this method without derivatisation fails to identify any other radioactive products with R_f values close to those of the localised product. This single step chromatographic method could lead to spurious results as has been shown in this study with the rat ovary. In the human placenta since there was no evidence of any products other than authentic estradiol and estrone, accumulative measurement of the estrogens produced comparable results as previously reported.[5] The product isolation method using appropriate solvent systems for different tissues with full characterisation of the products[9,13,14] nevertheless, remains the most definitive method to measure aromatisation.

In this study, using the validated tritiated water method, aromatase activity in the adult cyclic rat has been shown to peak at proestrus and rapidly returns to its basal level at estrus. Plasma estradiol concentrations were also found to parallel the aromatase activity. Although it has been reported that aromatase activity remains high at estrus,[15] immunocytochemical studies[16] have provided strong evidence showing that aromatase activity is at its highest in the granulosa cells of preovulatory follicles at proestrus which is in agreement with our results.

Therefore in conclusion, consistent results are obtained by measuring aromatisation in human placental microsomes using both the single step chromatographic product isolation method and the tritiated water method employing either [1β-^{3}H]-A_4 or [1β,2β-^{3}H]-A_4 as a substrate. On the other hand, although a product isolation method with full characterisation of the products remains the most definitive method, the tritiated water assay employing [1β-^{3}H]-A_4 as a substrate serves as a rapid and reliable alternative for the measurement of aromatisation in the rat ovarian 1,000 g supernatant.

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