Gas Chromatography-Mass Spectrometric Determination of Activity of Human Placental Aromatase Using 16 α -Hydroxyandrostenedione as a Substrate

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Aromatization of 16 α -hydroxyandrostenedione (16 α -OH AD) with aromatase in human placental microsomes was studied by gas chromatography-mass spectrometry (GC-MS) using [2,4,6,6,9 α ,16 β ,17 α -²H₇]estriol as an internal standard. 16 α -OH AD was incubated with the microsomes in the presence of NADPH in air. The metabolite was extracted with ethyl acetate and treated with NaBH₄. The reduced product, estriol, was isolated by Sep-Pak C₁₈ cartridge and then analyzed as the tris(trimethylsilyl)ether by a GC-MS (EI mode). The production of estriol was dependent upon protein concentration and incubation time. Apparent K_m and V_{max} values of the microsomal aromatase for 16 α -OH AD were 568 nM and 25.5 pmol/min/mg protein, respectively. In this assay, aromatase activity, estriol formation, could be determined at a level as low as 1 pmol/min/mg protein. Aromatase inhibitors, 4-hydroxy- and 6-oxo-androstenediones, prevented the estriol formation in a competitive manner with 25 and 30 nM of apparent K_i values, respectively.

Key words aromatase assay; 16 α -hydroxyandrostenedione; aromatization; human placental microsome; gas chromatography-mass spectrometry

Aromatase is a cytochrome P-450 enzyme responsible for the conversion of the 4-en-3-one steroids androstenedione (AD) and its 16 α -hydroxy derivative (16 α -OH AD) into the estrogens estrone and 16 α -hydroxyestrone, respectively^{1–3)} (Fig. 1). The aromatization process is thought to proceed *via* three sequential oxygenations at C-19 of the androgens, the eventual loss of the angular methyl group at C-19 and the elimination of the 1 β - and 2 β -hydrogens, resulting in the aromatization of the A-ring of the androgen molecules to form the estrogens.^{4,5)} It is now believed that a single enzyme species catalyzes the aromatization independent of the difference in D-ring substitution of the androgens in all tissues.^{2,3,6)} Inhibitors of aromatase are valuable as therapeutic agents in the treatment of estrogen-dependent breast cancer.⁷⁾ The human placenta provides a plentiful source of aromatase for the testing of the inhibitors along with the isolation of the enzyme. 16 α -OH AD rather than AD is a principal precursor of estrogen production in the placenta.^{2,3,8)} Thus, aromatase inhibitors developed for assay of the 16 α -OH AD aromatization may be more effective against the clinical application in the management of breast cancer, compared to those currently developed for assay of the AD aromatization.⁹⁾ 16 α -OH AD prevents the AD aromatization in a non-competitive manner, suggesting that there would be two binding sites in the active site of aromatase.^{10,11)} Considering the substrate specificity of aromatase along with the estrogen production in the placenta, it is quite possible that the determination of the aromatase activity using 16 α -OH AD as a substrate may play a critical role in not only the study of the aromatase function but also the development of the aromatase inhibitor.

In connection with work in our laboratory on spatial aspects of the active site and catalytic function of aromatase, we were interested in the 16 α -OH AD aromatization. This paper describes the development of a gas chromatography-mass spectrometry (GC-MS) method with a high sensitivity for assay of the 16 α -OH AD aromatization.

MATERIALS AND METHODS

16 α -OH AD,^{12,13)} 4-hydroxyandrostenedione (4-OH AD),¹⁴⁾ and 6-oxoandrostenedione (6-oxo AD)¹⁵⁾ were synthesized by previously reported methods. [2,4,6,6,9 α ,16 β ,17 α -²H₇] Estriol (d₄, 1%; d₅, 4%; d₆, 14%; d₇, 81%) was also prepared with the known method.¹⁶⁾ NADPH was purchased from Kohjin Co., Ltd. (Tokyo, Japan), NaBH₄ from Aldrich (Milwaukee, WI, U.S.A.) and *N,O*-bis(trimethylsilyl)trifluoroacetamide from Tokyo Kasei Kogyo Co. (Tokyo, Japan).

Enzyme Preparation Human term placental microsomes (sedimented after 60 min at 105000 *g*) were obtained as described by Ryan.¹⁷⁾ They were washed once with 0.5 mM dithiothreitol solution, lyophilized, and stored at –20 °C. No significant loss of aromatase activity was observed for three months.

Aromatization Studies with GC-MS Incubation experiments were carried out principally according to the previous method.¹⁸⁾ Briefly, a mixture of 4 μ M of 16 α -OH AD, 300 μ M of NADPH, 200 μ g protein of the placental microsomes, and 50 μ l of MeOH in 2.05 ml of 67 mM phosphate buffer solution (pH 7.4) was incubated at 37 °C in air. After 30 min of incubation, 100 ng of an internal standard, [²H₇]estriol, was added to each incubation mixture and extracted with EtOAc. The extract was reduced with NaBH₄, then placed in a Sep-Pak cartridge (Waters, Milford, MA, U.S.A.). The steroid fraction was eluted with 80% MeOH. The recovery rates for estriol and 16 α -hydroxyestrone with these procedures were in the range between 70 and 75%. In the above experiments, 100, 200, and 300 μ g of the microsomal protein and 10, 20, and 30 min of the incubation time were also employed to determine the linearity of the aromatization rate.

For the kinetic analysis, the incubation mixture contained 0.33, 0.50, 1.0, and 4 μ M of 16 α -OH AD, 200 μ g of the microsomal protein, 50 μ l of methanol, and the phosphate buffer solution (pH 7.4), in a 30 ml Erlenmeyer flask in a total volume of 2.1 ml. The mixture was incubated at 37 °C for 30 min.

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Table 1. Aromatization of 16 α -OH AD and [1 β -³H]16 α -OH AD with Human Placental Microsomes and Their Kinetic Analysis

Method	Substrate	Aromatization rate, ^{a)} pmol/min/mg protein	Aromatization kinetics ^{b)}	
			$V_{\max}$, pmol/min/mg protein	K_m , nM
GC-MS ^{c)}	16 α -OH AD	22.4 $\pm$ 0.57	25.5	568
Radiometric ^{d)}	[1 β - ³ H]16 α -OH AD	23.5 $\pm$ 0.97	28.9	590

a) 4 μ M of 16 α -OH AD or [1 β -³H]16 α -OH AD and 200 μ g of protein from human placental microsomes were used. $n=4$. b) Various concentrations of 16 α -OH AD or [1 β -³H]16 α -OH AD and 200 μ g of protein were used. Each value represents the mean of two determinations which varied by less than 5% of the mean. c) Estriol produced by NaBH₄ reduction of the aromatized product was analyzed by GC-MS method using [2H₇]estriol as an internal standard. d) The estrogen produced was obtained by measuring an amount of ³H₂O released in the incubation medium during aromatization of the tritiated substrate.

For the inhibition study, the incubation mixture contained 1.1, 2.3, and 3.7 μ M of 16 α -OH AD, various concentrations of inhibitors, 4-OH AD and 6-oxo AD, and 200 μ g of the microsomal protein, 50 μ l of methanol and the phosphate buffer solution (pH 7.4) in a total volume of 2.1 ml. The incubation was done as described above.

When [1 β -³H]16 α -OH AD was used as the substrate in the kinetic and inhibition studies, the aromatization rate was determined by measuring the amount of tritiated water released from the labeled substrate into the incubation medium during aromatization, by the method previously reported.¹⁸⁾ The incubations were carried out under the same conditions as those employed in the GC-MS experiments.

Derivatization of the Aromatized Product for GC-MS Analysis The aromatized product, estriol, was converted into the tris(trimethylsilyl) (tris-TMS) ether with *N,O*-bis-(trimethylsilyl)trifluoroacetamide according to the previous method.¹⁹⁾

GC-MS A Finnigan MAT SSQ GC-MS instrument (San Jose, CA, U.S.A.) was used, and the GC-MS conditions were the same as those previously used for the 6-alkylestradiols.^{19,20)} Quantitative analysis of estriol was performed with a selected ion monitoring method (electron impact mode); the molecular ion (M^+ , m/z 504) was the base ion. Then, the amount of estriol produced was obtained by the relative abundance of the molecular ion of the product to that (M^+ , m/z 511) of the internal standard, [2H₇]estriol. Retention times: 17.50 min for tris-TMS of estriol, and 17.45 min for tris-TMS of [2H₇]estriol.

RESULTS AND DISCUSSION

The aromatization study on 16 α -OH AD was initially studied to determine the aromatization rate of 16 α -OH AD by human placental aromatase. It was postulated that the aromatized product would be a mixture of 16 α -hydroxyestrone and its 17 β -reduced estrogen, estriol, produced by the action of 17 β -hydroxysteroid dehydrogenase in the incubation.^{8,21)} Thus, the aromatization product was converted into estriol by treatment with NaBH₄²¹⁾ in the experiment, which was then purified and analyzed as the tris-TMS ether by GC-MS. The aromatization rate of 16 α -OH AD, at a concentration of 4 μ M, increased linearly with increasing amounts of the placental microsomes (up to 300 μ g of protein); furthermore, the conversion of the substrate increased linearly with incubation time (up to 30 min). The aromatization rate was first determined in the experiment with a 30 min incubation time and 200 μ g protein of the placental microsomes. The aromatization rate obtained by the GC-MS method was almost the

Fig. 1. Structures of 16 α -Hydroxy Steroids and Aromatase Inhibitors

same as that obtained by the previously reported radiometric assay method,¹⁸⁾ using [1 β -³H]16 α -OH AD as a substrate (Table 1). The aromatization of 16 α -OH AD was then analyzed kinetically. When the concentration of the substrate was changed to between 0.33 and 4 μ M, a typical saturation curve was obtained. A Lineweaver-Burk plot of the velocity against the concentration of the substrate gave the apparent K_m and $V_{\max}$ values, which corresponded well, respectively, to those obtained by the radiometric assay using [1 β -³H]16 α -OH AD (Table 1). In this GC-MS assay method, about 1 pmol estriol formation/min/mg protein could be reliably measured ($S/N>10$).

To validate the GC-MS method, 16 α -OH AD was employed as a substrate of aromatase for the inhibition study. 4-OH AD and 6-oxo AD (Fig. 1), typical suicide substrates of aromatase,^{22,23)} blocked the 16 α -OH AD aromatization with the placental microsomes in a competitive manner, respectively. The inhibition kinetics obtained for the two inhibitors by the GC-MS method were comparable to those obtained by the radiometric assay (Fig. 2 and Table 2).

The present results indicated that the non-radioisotopic GC-MS method developed in this study is sensitive enough and useful for the assay of the 16 α -OH AD aromatization with human placental aromatase, as an alternate method to the radiometric method using commercially non-available [1 β -³H]16 α -OH AD as a substrate.

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Table 2. Inhibition of 16 α -OH AD and [1 β -³H]16 α -OH AD Aromatization by Aromatase Inhibitors^{a)}

Inhibitor	IC ₅₀ ^{b)} nM		K _i ^{c)} nM (Inhibition type ^{d)})	
	GC-MS method	Radiometric method	GC-MS method	Radiometric method
4-OH AD	125	118	25 (competitive)	28 (competitive)
6-Oxo AD	150	162	30 (competitive)	32 (competitive)

a) Each value represents the mean of two determinations which varied by less than 5% of the mean. b) 4 μ M of 16 α -OH AD (GC-MS method) or [1 β -³H]16 α -OH AD (radiometric method), 200 μ g of the microsomal protein, and 30 min incubation time were used. c) An apparent K_i value was obtained by Dixon Plot. d) Inhibition type was determined by Lineweaver–Burk plot.

Fig. 2. Lineweaver–Burk Plot of the Inhibition of Human Placental Aromatase by 6-Oxo AD with 16 α -OH AD as a Substrate

Each point represents the mean of two determinations which varied by less than 5% of the mean. The inhibition experiment with 4-OH AD gave an essentially similar result to that for 6-oxo AD (data not shown).

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