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## Note

Clinical analysis of steroids

XXXI*. Assay of oestradiol 17-sulphate 4-hydroxylase activity by high-performance liquid chromatography with electrochemical detection

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Since the discovery of 2-methoxyoestrone [1, 2], the main metabolism of oestrogens has long been recognized as the introduction of an oxygen function at the C-2 position to produce 2-hydroxyoestrogens. Later, a similar biological hydroxylation was found to occur also at the C-4 position, by which another type of catechol, 4-hydroxyoestrogens, are formed [3]. These catechol oestrogens are now being widely investigated in numerous laboratories, because they are not just inactive metabolites but act physiologically as neurotransmitters [4–6].

Previously, we demonstrated the sulphate-specific, and sex-dependent 2-hydroxylation of oestradiol 17-sulphate (E-17-S) by rat liver microsomes with an NADPH-generating system [7]. Namely, liver microsomes from male rats converted E-17-S to 2-hydroxyoestradiol 17-sulphate (2-OH-E-17-S) as the sole product. In contrast, no such regulating effect was observed in the microsomes from female rats, and multiple kinds of hydroxylated products including a 2-hydroxylated metabolite were formed. Recently, this catechol formation from E-17-S was found to be not so specific because another type of catechol, 4-hydroxyoestradiol 17-sulphate (4-OH-E-17-S), was produced, although in extremely small amounts [8].

In a recent paper of this series, we reported an assay method for E-17-S 2-hydroxylase employing high-performance liquid chromatography (HPLC) [9]. Application of this HPLC method to induction studies has revealed that the 2-hydroxylation of E-17-S by rat liver microsomes is induced by

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*For Part XXX, see I. Yoshizawa and M. Kameyama, Chem. Pharm. Bull., 33 (1985) in press.

cytochrome P-450 inducer [10]. Whether such an induction could occur also for the 4-hydroxylation is an interesting problem to be investigated. For this purpose, it is necessary to establish an assay method for E-17-S 4-hydroxylase.

The present paper deals with an assay method for E-17-S 4-hydroxylase activity by HPLC with electrochemical detection (ED).

EXPERIMENTAL

*Materials*
E-17-S [11], 2-OH-E-17-S [12], 4-OH-E-17-S [8], and 2-methoxyoestradiol 17-sulphate [13], were prepared in this laboratory according to the known methods. Glucose 6-phosphate (G-6-P), NADP, and G-6-P dehydrogenase were obtained from Oriental Yeast (Osaka, Japan). Sep-Pak $C_{18}$ cartridges and column guards were obtained from Waters Assoc. (Milford, MA, U.S.A.) and Millipore (Bedford, MA, U.S.A.), respectively. All other reagents and solvents used were purchased commercially. Methanol and water used for elution of steroid conjugates through cartridges were bubbled with nitrogen gas to remove any dissolved oxygen, followed by addition of ascorbic acid (5 mg in each 100 ml).

*High-performance liquid chromatography*
HPLC was carried out using a Model 803 chromatograph equipped with an EC-8 electrochemical detector (Toyo Soda, Tokyo, Japan) at 0.8 V versus the Ag/AgCl reference electrode. A stainless-steel column (25 cm × 4.6 mm I.D.) packed with TSK-Gel ODS-120 A (5 $\mu$m) (Toyo Soda) was used and maintained at 40°C in a circulating water bath. A mixture of 0.5% ammonium dihydrogen phosphate (pH 3.0) and methanol (60:40, v/v) was used as mobile phase. The flow-rate of the mobile phase was 1.0 ml/min and the column pressure 130 kg/cm².

*Animals*
Wistar rats, ranging in age from 58 ± 7 days after birth for male (230—280 g) to 62 ± 7 days after birth for female (190—220 g) were used. All the animals were starved for 18 h prior to decapitation.

*Preparation of microsomes*
Rat liver microsomes were prepared by the method described previously [7, 8, 10]. Microsomal protein was determined by the method of Lowry et al. [14] using bovine serum albumin as reference standard.

*Assay procedure*
The standard incubation was carried out using the following conditions. Ice-cold reaction vessels contained microsomal protein (0.5 ml), an NADPH-generating system (NADP, 0.5 m$M$; G-6-P, 5 m$M$; magnesium chloride, 5 m$M$; G-6-P dehydrogenase, 0.6 U/ml; potassium chloride, 90 m$M$; EDTA, 0.1 m$M$; and E-17-S, 0—400 $\mu M$). The mixture was diluted with Tris—HCl buffer (pH 7.4, 50 m$M$) to 3.0 ml as a final volume and was incubated for 0—60 min at 37°C under aerobic conditions. The reaction was terminated by heating the

incubation vessels in boiling water for 1 min, followed by addition of ascorbic acid (5 mg) as antioxidant and a known amount of 2-methoxyoestradiol 17-sulphate as internal standard (1.0—10 μg), and finally was diluted with 10 ml of water. For the control experiment, the incubation was performed using boiled microsomes (100°C for 1 min) using the same procedure as described above.

The incubation mixtures were centrifuged at 1500 $g$ for 20 min, and the precipitates were suspended in water and again centrifuged. The combined supernatants were passed through Sep-Pak $C_{18}$ cartridges. After washing with 2.0 ml of water, the conjugated fractions were obtained by elution with methanol (4.0 ml). The eluates were passed through column guards and the methanolic filtrates were evaporated under a nitrogen stream at 40°C to give the residues, which were subjected to HPLC as methanolic solutions. The enzyme activity of 2-hydroxylase was determined by a slight modification of the method described previously [9].

RESULTS AND DISCUSSION

The methanolic eluates of the incubation mixture passed through Sep-Pak $C_{18}$ cartridges were subjected to HPLC, the results of which are compared with the chromatogram of authentic specimens in Fig. 1. Three peaks (1, 2 and 3) in both sexes are coincident with those of the authentic conjugates, E-17-S, 2-OH-E-17-S and 4-OH-E-17-S, respectively. A group of peaks (4) composed of three kinds of peaks appeared when female rats were used, and they were identified as E-17-S metabolites hydroxylated at C-6β, C-7β and C-16α positions [7].

As described previously [7, 8], these hydroxylations were found to proceed without removal of the conjugated group at C-17 of the substrate used. Since control experiments using boiled microsomes showed no product formation, these hydroxylations should occur enzymatically.

Because 2-OH-E-17-S was obtained in a fairly large amount and could be measured by ultraviolet detection as well as the more sensitive ED, the assay method for E-17-S 2-hydroxylase was established without difficulty [9]. The amount of 4-OH-E-17-S formed from E-17-S, on the other hand, was extremely small. The minimum amount of 4-OH-E-17-S produced in a series of kinetic studies was about 5 ng per incubation sample, and one-tenth of these samples was injected for HPLC. The amount of the catechol injected is, thus, nearly equal to the detection limit of 4-OH-E-17-S by ED (0.2 ng, signal-to-noise ratio = 3.5 at 1 nA full scale) (Fig. 1d). Therefore, ED is absolutely necessary for performing enzymatic studies on E-17-S 4-hydroxylase.

Since peak 3 was confirmed to be composed solely of 4-OH-E-17-S [8], development of a method for quantification of this catechol by determining the peak height became possible. The calibration curve for 4-OH-E-17-S was constructed by plotting the peak height of the material to that of the internal standard against the amount of the former, and a satisfactory linearity was observed in the range of 1—100 ng of the catechol.

In order to confirm the validity of the present method for the determination of 4-OH-E-17-S, a recovery test was undertaken using authentic sample. A known amount of the conjugate was added to the medium, and the conjugate
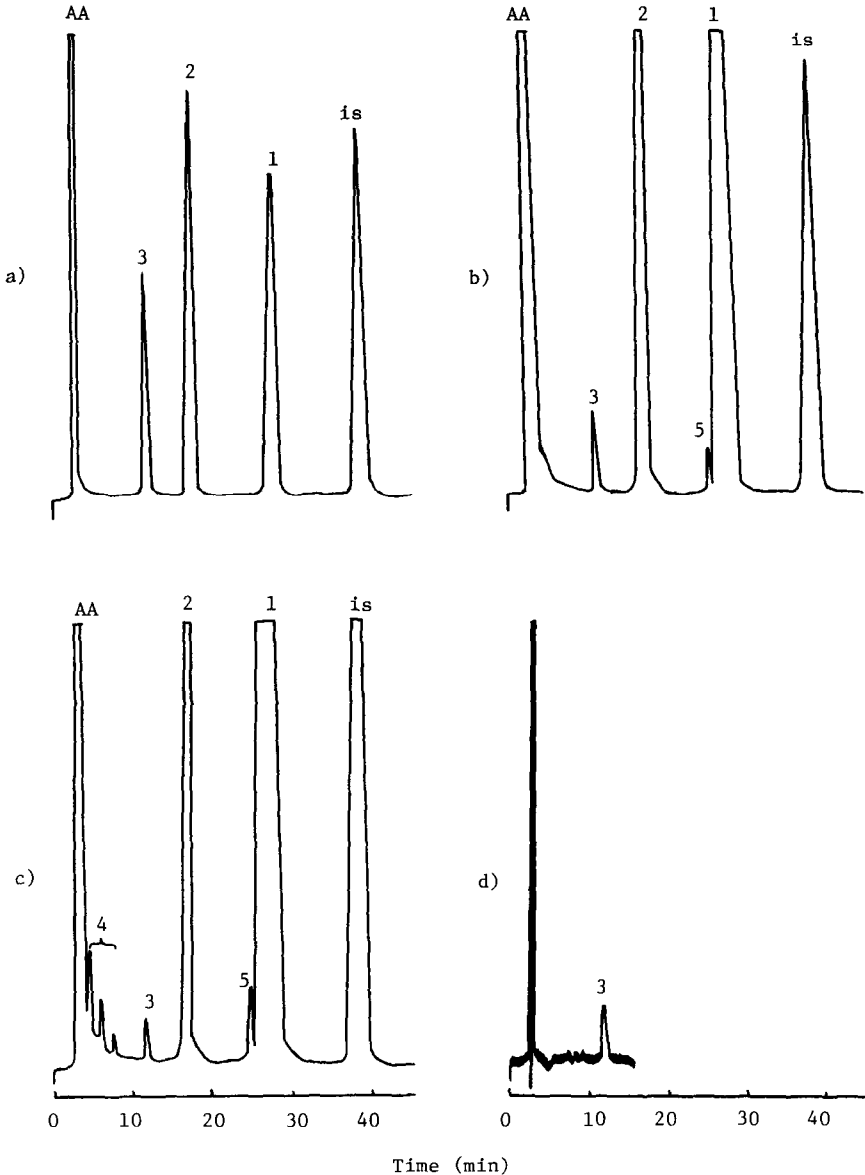
Fig. 1. High-performance liquid chromatograms of authentic conjugates (a), incubation products of oestradiol 17-sulphate of liver microsomes from male (b) and female (c) rats, and detection limit of 4-hydroxyoestradiol 17-sulphate (d) using electrochemical detection. Peaks: 1, oestradiol 17-sulphate; 2, 2-hydroxyoestradiol 17-sulphate; 3, 4-hydroxyoestradiol 17-sulphate; 4, group composed of metabolites of oestradiol 17-sulphate hydroxylated at $6\beta$, $7\beta$ and $16\alpha$; 5, peak derived from microsomes; is, 2-methoxyoestradiol 17-sulphate (internal standard); AA, ascorbic acid.

recovered through the whole clean-up procedure was determined. The results (Table I) indicate that 4-OH-E-17-S is recovered to a satisfactory extent.

Some kinetic parameters of E-17-S 4-hydroxylase were measured by the present method. Fig. 2 shows the influence of the incubation time (a), enzyme concentration (b) and hydrogen ion concentration (c) on the production of

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TABLE I

RECOVERIES OF 4-HYDROXYOESTRADIOL 17-SULPHATE FROM THE INCUBATION MEDIUM AND AFTER THE WHOLE CLEAN-UP PROCEDURE

Steroid was dissolved in the incubation medium (3.0 ml) and the mixture was immediately heated for 1 min in boiling water, followed by the same treatment as described in the text. Each value is expressed as the mean % ± S.D. ($n = 6$).

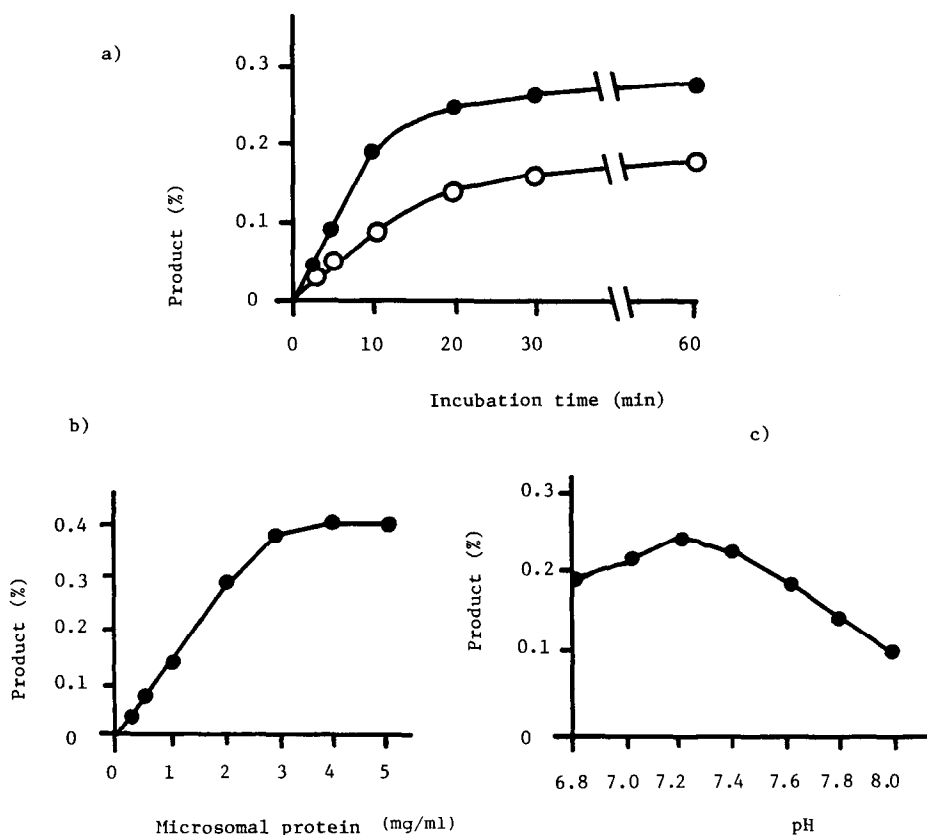| Amount added ($\mu$g) | Recovery (%) |
|---|---|
| 10 | 99.1 ± 3.2 |
| 5 | 98.0 ± 2.4 |
| 2 | 97.7 ± 2.0 |
| 0.2 | 100.1 ± 2.9 |



Fig. 2. Effect of incubation time (a), microsomal protein concentration (b) and hydrogen ion concentration (c) on the 4-hydroxylation of oestradiol 17-sulphate by male rat liver microsomes. Oestradiol 17-sulphate (200 $\mu M$) was incubated with an NADPH-generating system at 37°C at the following conditions: (a) pH 7.4 using protein concentration of 2.0 mg/ml (•) and 1.0 mg/ml (○); (b) pH 7.4 for 30 min; (c) for 30 min using a protein concentration of 2.0 mg/ml. Each point represents the mean value of six experiments.

4-OH-E-17-S by male rat liver microsomes. The enzyme activity was linear up to 10 min incubation and up to 2.0 mg/ml protein. The effect of pH on the product formation was studied, and the optimum pH was shown to be about 7.2. Similar results were obtained when female rats were used.

The enzyme kinetics of E-17-S 4-hydroxylase from the microsomes of male rat liver follow classical Michaelis—Menten kinetics producing a Lineweaver—Burk plot (Fig. 3). Analogous results were obtained from the experiments with female rat liver microsomes.
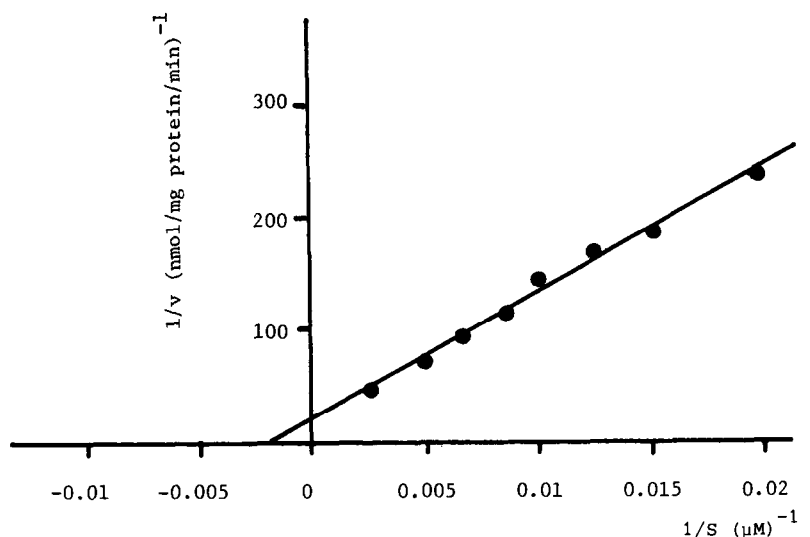


Fig. 3. Lineweaver—Burk plot for oestradiol 17-sulphate 4-hydroxylase of male rat liver microsomes.

TABLE II

COMPARISON OF APPARENT $K_m$ ($\mu M$) AND $V_{max}$ (nmol/mg PROTEIN/min) VALUES FOR OESTRADIOL 17-SULPHATE 2- AND 4-HYDROXYLASES IN RAT LIVER MICROSOMES

| Sex | 4-Hydroxylase | | 2-Hydroxylase | |
|-----|-----|-----|-----|-----|
| | $K_m$ | $V_{max}$ | $K_m$ | $V_{max}$ |
| Male | 540 | 0.047 | 185 | 1.35 |
| Female | 570 | 0.050 | 200 | 0.41 |

The E-17-S 4-hydroxylase activity was measured from a series of six separately prepared samples using the same rat liver microsomes. Table II shows a comparison of the apparent $K_m$ and $V_{max}$ values for E-17-S 2- and 4-hydroxylases in rat liver microsomes from either sex under these conditions. From these results, there seems to be some difference between the two enzymes, especially in their $K_m$ and $V_{max}$ values. A sex difference between the two enzymes is observed in the case of 2-hydroxylation. Such a sex difference of 2-hydroxylation was also observed in the metabolism of free oestradiol [15].

The $K_m$ and $V_{max}$ values of 2-hydroxylase in Table II are different from

those reported in the previous paper [9], the reported values being 85.5 $\mu M$ and 0.64 nmol per mg protein per min in the male rats, and those in female rats were 117 and 0.19, respectively. The discrepancy between the previous and the present results may be attributable to the age of the animals studied. This is estimated by the recent report by Theron et al. [16] who showed that the enzyme kinetics of oestradiol 2-hydroxylase undergo a dramatic change during the development of the animals. Since we have used animals of almost the same age in the present experiments, in contrast to the previous report [9], the results in Table II on 2-hydroxylase should be more reliable. Further investigations of the influence of age on the 2- and 4-hydroxylation of E-17-S are needed.

Utilization of the present method in biological and endocrinological studies of the C-17 conjugated oestradiol will be the subject of a future communication.

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