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CLINICAL ANALYSIS ON STEROIDS

XXV*. ASSAY OF ESTRADIOL 17-SULFATE 2-HYDROXYLASE ACTIVITY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple method for the assay of the 2-hydroxylation enzyme of estradiol 17-sulfate in rat liver microsomes has been established by using reversed-phase high-performance liquid chromatography with electrochemical detection. The technique devised involves elution with 50 m*M* acetate buffer, pH 5.0—methanol (3:2, v/v) using an ODS SIL column and monitoring the potentials at 1.1 V vs. the silver/silver chloride reference electrode. The calibration curve of the relationship between the amounts of 2-hydroxyestradiol 17-sulfate (5—50 ng) injected and peak heights (cm) of the chromatogram was linear. By this method, some kinetic parameters of estradiol 17-sulfate 2-hydroxylase were measured.

INTRODUCTION

Catechol estrogens produced by hydroxylation at C-2 of female hormone estrogens are now recognized as physiologically potent metabolites in living animals [1—3]. This *ortho*-hydroxylation of phenol is also observed in the metabolism of synthetic estrogens such as diethylstilbestrol, and also in that of therapeutically so-called "antiestrogens" such as tamoxifen.

In a recent investigation [5] we demonstrated the sulfate-specific 2-hydroxylation of estradiol 17-sulfate (E-17-S) by liver microsomes from male rat. In contrast, no such regulating effect was observed in those from female

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rat, in which multiple kinds of hydroxylated products including 2-hydroxylated metabolite were formed. To investigate the characteristics of the 2-hydroxylase for E-17-S, it became necessary to establish the assay for the enzyme.

In the present paper we wish to report a simple method for assay of the 2-hydroxylase activity by determining the product, 2-hydroxyestradiol 17-sulfate (2-OH-E-17-S) by high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Materials

2-Hydroxyestradiol [6], 2-OH-E-17-S [7], 2-OH-E-17-S-2-methyl ether [8], 4-hydroxyestradiol [9], 6α- and 6β-hydroxyestradiols [10, 11], and 7α- and 7β-hydroxyestradiols [12], were prepared in this laboratory according to the known methods. E-17-S was prepared from estradiol by the method of Kirdani [13]. Estradiol and estriol were purchased from Steraloids (Wilton, NH, U.S.A.). 4-Nitroestrone and 15α-hydroxyestradiol were generous gifts from Dr. J. Fishman (The Rockefeller University, New York, NY, U.S.A.), to whom our thanks are due. Glucose 6-phosphate (G-6-P), NADP, NADPH, and G-6-P dehydrogenase, were obtained from Oriental Yeast, Inc. (Osaka, Japan). Sep-Pak C_{18} cartridges were obtained from Waters Associates (Milford, MA, U.S.A.). Methanol and water used for elution of steroidal conjugates through cartridges were bubbled with nitrogen to remove any dissolved oxygen, followed by addition of ascorbic acid (5 mg in each 100 ml). Peroxide-free ether was prepared and used for the solvolysis of the conjugates. All other reagents and solvents used were obtained commercially.

High-performance liquid chromatography

HPLC was carried out using a Model 803 chromatograph equipped with an EC-8 electrochemical detector at 1.1 V vs. the Ag/AgCl reference electrode (Toyo Soda, Tokyo, Japan). A stainless-steel column (30 cm × 4.0 mm I.D.) packed with TSK-Gel LS 410 ODS SIL (5 μm) (Toyo Soda) was used and maintained at 40°C in a circulating water bath. Preparative HPLC was carried out by the same machine using a column (30 cm × 7.5 mm, I.D.) packed with the same stationary phase, and a UV detector 1205-T (Toyo Soda) at 280 nm. The following solvent systems were used as mobile phase. System A: acetate buffer (50 m*M*, pH 5.0)—methanol (60 : 40, v/v); system B: acetate buffer (50 m*M*, pH 4.0)—methanol (45 : 55, v/v); system C: acetate buffer (50 m*M*, pH 4.0)—methanol (45 : 36, v/v).

Animals

Wistar rats were fed a synthetic diet. All the animals, weighing 200—300 g, were starved for 18 h prior to sacrifice.

Preparation of microsomes

Rat liver microsomes were prepared by the method described previously [5]. The 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 with 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*; $MgCl_2$, 5 m*M*; G-6-P dehydrogenase, 0.6 unit/ml), KCl (90 m*M*), EDTA (0.1 m*M*), and E-17-S (0—400 μM). 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 at 37°C under aerobic conditions. The mixture was incubated for 30 min except for the study of the time-course. 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 internal standard (approx. 0.5—20 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) with 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 conjugate fraction was obtained by elution with methanol (4.0 ml). The eluates were passed once through the membrane filter (0.8 μm) and the methanolic filtrates were evaporated under a nitrogen stream at 40°C to give the residues, which were subjected to HPLC using system A as mobile phase.

Separation and solvolysis of steroid conjugates

The incubation mixtures of ten experiments (substrate concentration: 200 μM) were passed through Sep-Pak C_{18} cartridges, and the combined methanolic eluate was dried under a nitrogen stream. The residue was subjected to preparative HPLC using solvent system A. Confirmation of the separation of these fractions was done by HPLC with electrochemical detection. Three separated fractions were submitted to solvolysis by the method of Burstein and Lieberman [15], with the modification of adding ascorbic acid to the ether and aqueous layers. After the addition of 4-nitroestrone (about 10 μg) as internal standard, each hydrolyzate was subjected to HPLC using systems B and C.

RESULTS

The methanolic eluates of the incubation mixture through Sep-Pak C_{18} cartridges were applied to HPLC, the results of which are shown in Fig. 1. Two peaks (1 and 2) in both sexes are coincident with those of authentic specimens, E-17-S and 2-OH-E-17-S, respectively. The formation of another peak (3, consisting of three kinds of peaks) was observed only when the female rat was used. Because the hydroxylated product of E-17-S is not available except 2-OH-E-17-S, it is necessary to confirm that peak 2 is composed of only 2-OH-E-17-S. To examine this, the following experiment was undertaken.

An incubation mixture was applied to preparative HPLC to separate the products corresponding to peaks 1, 2, and 3 in Fig. 1. Solvolysis of these fractions gave their hydrolyzates. Only 2-hydroxyestradiol was detected

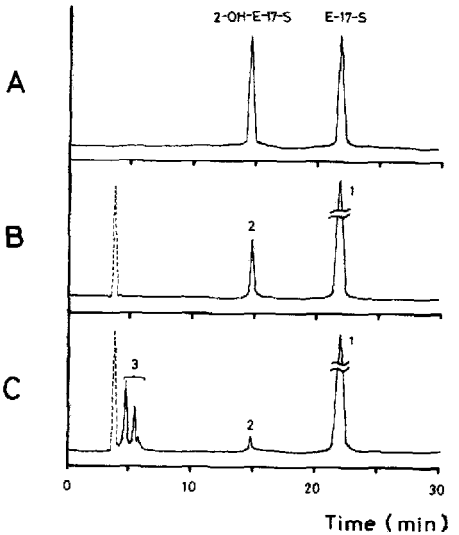

Fig. 1. Comparison of high-performance liquid chromatograms between the authentic conjugates (A) and incubation products of estradiol 17-sulfate with rat liver microsomes of male (B) and female (C) rats. Dotted lines are peaks of ascorbic acid added as antioxidant. E-17-S = estradiol 17-sulfate, 2-OH-E-17-S = 2-hydroxyestradiol 17-sulfate.

TABLE I

COMPARISON OF THE RELATIVE RETENTION TIMES OF AUTHENTIC STEROIDS AND THE HYDROLYZATES OF INCUBATION PRODUCTS OF ESTRADIOL 17-SULFATE

Compound	Solvent system	
	B	C
Estradiol	1.08	0.98
2-Hydroxyestradiol	0.64	0.51
4-Hydroxyestradiol	0.60	0.44
6α-Hydroxyestradiol	0.24	0.13
6β-Hydroxyestradiol	0.33	0.22
7α-Hydroxyestradiol	0.24	0.13
7β-Hydroxyestradiol	0.25	0.14
15α-Hydroxyestradiol	0.23	0.12
16α-Hydroxyestradiol (estriol)	0.31	0.19
Hydrolyzate of peak 1	1.09	0.98
Hydrolyzate of peak 2	0.64	0.51
Hydrolyzate of peak 3	0.25 0.31 0.33	0.14 0.18 0.22
4-Nitroestrone	1.00 (27.40 min)	1.00 (60.80 min)

in the hydrolyzate of peak 2, and only estradiol in that of peak 1, whereas in the hydrolyzate of peak 3, 6β- and 7β-hydroxyestradiols, and estriol were found (Table I). It was confirmed, therefore, that peak 2 is composed of only 2-OH-E-17-S, and peak 3 is a mixture of the 17-sulfates of 6β-, 7β-, and

16α-hydroxylated estradiols. Peak 1 was composed of only the substrate. No 4-hydroxyestradiol was detected in any hydrolyzate, although this catechol is known to be produced from estradiol in human tissues [16].

From the above results, it can be seen that the peak height of peak 2 of the incubation products depends on the amount of 2-OH-E-17-S produced. Thus, the quantification of 2-OH-E-17-S by HPLC became possible. Development of a method for the quantification of 2-OH-E-17-S was then undertaken. The calibration curve was constructed by plotting the peak height of 2-OH-E-17-S to that of the internal standard against the amount of the former, and a satisfactory linearity was observed in the range of 5—100 ng of the catechol. An analogous result was obtained for the substrate, E-17-S.

In order to confirm the validity of the present method for the determination of 2-OH-E-17-S, the recovery test was carried out using authentic sample. A known amount of the conjugates was added to the incubation medium, and the conjugates recovered through the whole clean-up procedure were determined. It is evident from the data in Table II that 2-OH-E-17-S was recovered to a satisfactory extent.

TABLE II

RECOVERIES OF ESTROGEN CONJUGATES* FROM THE INCUBATION MEDIUM AND TAKEN THROUGH THE WHOLE CLEAN-UP PROCEDURE

Each steroid was dissolved in the incubation medium (3.0 ml) and the mixtures were immediately heated for 1 min in boiling water followed by the same treatment as described in the assay procedure

Added amount (μmol)	Recovery (%)	
	E-17-S	2-OH-E-17-S
0.05	88.01 ± 2.94**	89.87 ± 2.31
0.1	87.67 ± 2.61	90.88 ± 2.56
0.5	88.56 ± 1.63	94.02 ± 1.12
1.0	90.72 ± 1.35	91.17 ± 0.94

*E-17-S = estradiol 17-sulfate; 2-OH-E-17-S = 2-hydroxyestradiol 17-sulfate.
**Mean ± S.D. (n = 6).

Some kinetic parameters of E-17-S 2-hydroxylase were also measured by the present method. Fig. 2 shows the influence of the incubation time and that of the enzyme concentration upon the production of 2-OH-E-17-S by male rat liver microsomes. The enzyme activity was linear up to 30 min incubation and up to 2.0 mg of protein. Fig. 3 shows the effect of substrate concentration upon product formation. Analogous results were obtained from the experiment with female rats.

The enzyme kinetics of E-17-S 2-hydroxylase from the microsomes of the male rat follows classical Michaelis—Menten kinetics producing a linear Lineweaver—Burk plot (Fig. 4). Analogous results were obtained when female rat liver microsomes were used. The apparent K_m values for E-17-S under these conditions were 85.5 and 117 μM for the 2-hydroxylase of male and

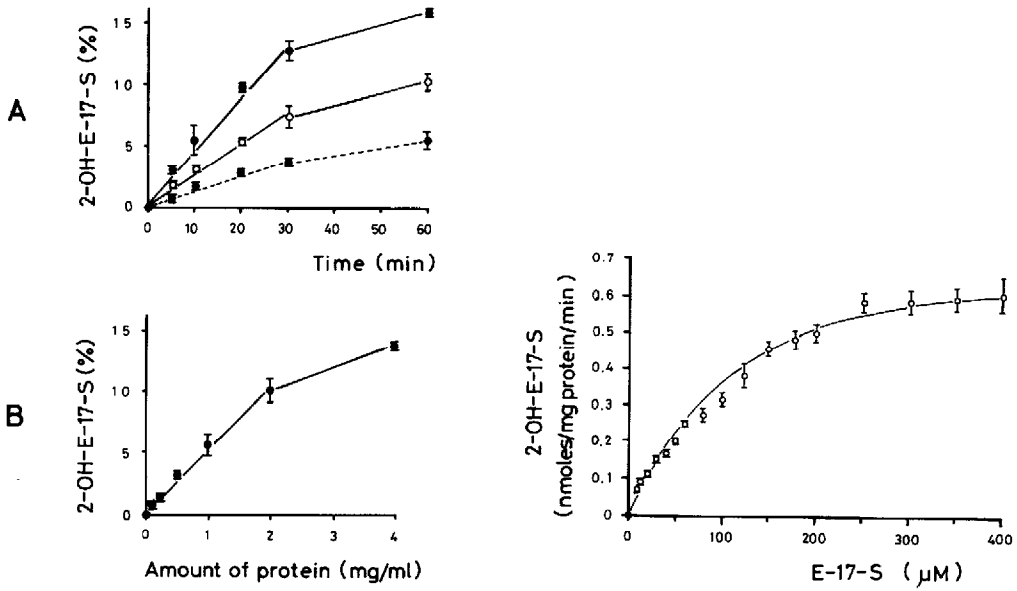

Fig. 2. (A) Effect of incubation time with different microsomal protein concentrations: (•—•), 2 mg/ml; (○—○), 1 mg/ml; (•---•), 0.5 mg/ml. (B) Effect of microsomal protein concentration upon the product formation. Substrate concentration = 200 μM, n = 5. Incubation time in B = 30 min.

Fig. 3. Effect of substrate concentration on estradiol 17-sulfate 2-hydroxylase activity of male rate liver microsomes. E-17-S = estradiol 17-sulfate, 2-OH-E-17-S = 2-hydroxy-estradiol 17-sulfate.

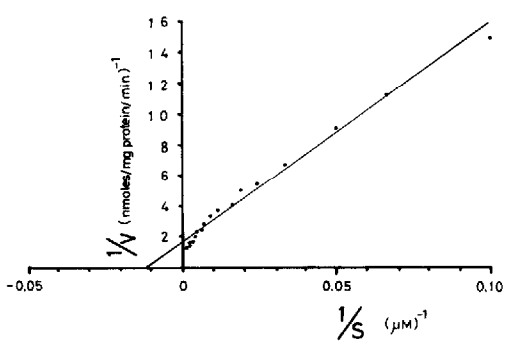

Fig. 4. Lineweaver—Burk plot for estradiol 17-sulfate 2-hydroxylase of male rat liver microsomes.

female rats, respectively. The V_{max} values for freshly prepared microsomes were 0.64 and 0.19 nmol/mg protein per min, for male and female rats, respectively.

DISCUSSION

Many skeletal carbons or even the angular methyl group are oxidized in the metabolism of estrogen in several species including humans, such as at positions 2, 4, 6, 7, 11, 14, 15, 16, and 18 [17, 18]. Hydroxylation at C-2, how-

ever, is the major pathway in estrogen metabolism. Our previous results [5] on rat liver microsomal hydroxylation of E-17-S demonstrated that the hydroxylation was sex-dependent, and that the 2-hydroxylation by male rat was sulfate-specific. Clarification of the enzyme is, therefore, important endocrinologically in estrogen metabolism. In the present study, the assay of the 2-hydroxylase by HPLC became possible. This is due to the fact that the separated peak corresponding to 2-OH-E-17-S was shown to be composed solely of this catechol.

In spite of the numerous reports on the formation of 4-hydroxylated estrogen in human and other animals [16, 19], the present result demonstrates that no detectable amount of 4-hydroxylated product was found.

The apparent K_m value obtained for E-17-S by male rat liver microsomes was 85.5 μM, that is a lower value than that for free estradiol: 2.2 μM [20], 2.0 μM [21], and 39.2 μM [22]. The difference in the value between free and 17-sulfoconjugated estradiols may be reasonable considering the characteristics of the microsomal enzyme.

The sex difference in kinetic parameters, especially the K_m value, as observed in the present paper, indicates that the hepatic 2-hydroxylation enzyme is different between male and female rats. An analogous results has been recently reported, although the result was on the 2-hydroxylation of free estradiol [20].

Several authors have already reported the assay of estradiol 2-hydroxylase using a radioenzymatic method [21], and a radiometric procedure [22, 23]. The present method for estrogen 2-hydroxylase using HPLC may be applied also to free estrogen metabolism because of its simplicity and greater speed.

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