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Studies on High-Performance Liquid Chromatography with Electrochemical Detection. The pH-Dependency of Enzymic Sulfation of Catechol Estrogens[1)]

KAZUTAKE SHIMADA, MASAMI HARA, and TOSHIO NAMBARA*

Pharmaceutical Institute, Tohoku University, Aobayama, Sendai 980, Japan

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In vitro sulfation of catechol estrogens with guinea pig and rat liver homogenates has been investigated by means of high-performance liquid chromatography with electrochemical detection. When incubated in a neutral medium (pH 7.4) with the liver 105000*g* supernatant fortified with 3′-phosphoadenosine-5′-phosphosulfate, 2-hydroxyestrone and 4-hydroxyestrone were transformed principally into the 2- and 4-sulfates, respectively. The 3-sulfate was formed, and decreasing amounts of the 2- and 4-sulfates were obtained, as the pH of the incubation medium was decreased. The pH effect on directive glucuronidation was also examined with catechol estrogens. The product ratio of the isomeric ring A glucuronides formed was not affected by the pH of the incubation medium.

Keywords——high-performance liquid chromatography; electrochemical detection; catechol estrogen; guinea pig liver; rat liver; positional isomer ratio; pH-dependent sulfation

In a previous paper,[2)] we reported a study of the *in vitro* metabolic conjugation of catechol estrogens, 2-hydroxyestrone and 4-hydroxyestrone by means of high-performance liquid chromatography (HPLC) with electrochemical detection (ECD). Glucuronidation of the two catechols with the rat and human liver 1500*g* supernatants in the presence of uridine-5′-phosphoglucuronic acid provided the 2- and 4-glucuronides, respectively. In contrast, incubation of catechol estrogens with the guinea pig liver 1500*g* supernatant yielded both isomeric ring A monoglucuronides. These substrates underwent sulfation with the rat liver cytosol fraction fortified with 3′-phosphoadenosine-5′-phosphosulfate (PAPS), providing the 2- and 4-sulfates. This investigation, however, has not yet been extended to other species.

Recently, the multiplicity of aryl sulfotransferase was suggested and purification of the enzyme was undertaken by several groups.[3-5)] Species difference may be observed in the sulfation of catechol estrogens, and hence the properties of the enzyme involved are of interest. In this study, we have attempted to clarify the nature of *in vitro* sulfation of catechol estrogens by guinea pig liver in comparison with that by rat liver.

Materials and Methods

Chemicals and Reagents——All catechol estrogens,[6)] PAPS, and 3′,5′-diphosphoadenosine (PAP)[7)] were prepared in these laboratories. Other chemicals and reagents used were of analytical grade. Buffer solutions of 0.1 M phosphate (pH 4.9, 5.9, 7.0, 7.4, 8.0, and 9.1) and 0.1 M Tris-HCl (pH 7.4) were used for incubation studies. The pHs of other phosphate buffers were adjusted with H_3PO_4 or NaOH. A trace amount of ascorbic acid was added to the incubation medium (pH >9.1) to prevent oxidative decomposition of catechol estrogens. The results of incubation studies were independent of the kind of buffer solutions and the presence of ascorbic acid.

HPLC——HPLC was performed on a Toyo Soda 803 A liquid chromatograph equipped with an EC 8 electrochemical detector (Toyo Soda Co., Tokyo). The applied potential was set at +0.9 V *vs.* Ag/AgCl reference electrode. A TSKgel ODS-120T (5 μm) column (25 cm × 0.4 cm i.d.) (Toyo Soda Co.) was employed at a flow rate of

1 ml/min. The detection limit (S/N = 2 at 2 nA full scale) was 1 ng for catechol estrogen ring A monosulfate.

Enzyme Preparation——A Beckman L5-65 ultracentrifuge was used for the preparation of subcellular fractions. Hartley strain male guinea pigs weighing 400—500 g were fasted overnight before sacrifice. Fresh liver was homogenized in ice-cold 0.25 M sucrose solution with a Teflon-glass homogenizer to provide a final concentration of 20% (w/v). The homogenate was centrifuged for 15 min at $600 \times g$, and the supernatant was centrifuged successively at $8000 \times g$ for 10 min, at $15000 \times g$ for 10 min, and at $105000 \times g$ for 60 min to provide the mitochondria, lysosome and microsome fractions, respectively. In a similar fashion, the fresh liver homogenate (20% solution in 0.25 M sucrose) from male Wistar rats (200—300 g) was fractionated, and the subcellular fractions were used for incubation studies. Protein was determined by the method of Lowry *et al.*[8] using bovine serum albumin as a reference.

Assay Procedure for Enzymic Sulfation——The standard assay medium (4 ml) contained substrate (175 nmol in 0.1 ml of MeOH), PAPS (350 nmol in 1 ml of 1 mM Tris-HCl buffer (pH 8.7)), enzyme preparation (1 ml of 105000 g supernatant: 7 mg protein) and 1.9 ml of 0.1 M Tris-HCl buffer (pH 7.4) containing 0.1 mM dithiothreitol. After preincubation for 5 min the mixture was incubated under aerobic conditions at 37 °C for 120 min. After addition of *p*-dimethylaminobenzoic acid as an internal standard (IS), the incubation mixture was deproteinized by heating it, and centrifuged. The supernatant was separated and percolated through an Amberlite XAD-4 column (15 cm × 1 cm i.d.). The column was washed with water (10 ml), and the desired fraction was eluted with MeOH (5 ml), evaporated down *in vacuo* and then subjected to HPLC/ECD using 0.5% $NH_4H_2PO_4$ (pH 3.0)–tetrahydrofuran–acetonitrile (16 : 2 : 3 and 16 : 3 : 2 for 4-hydroxyestrogens and 2-hydroxyestrogens, respectively) as the mobile phase.[9] A calibration graph was constructed by plotting the ratio of the peak height of catechol estrogen sulfate to that of IS against the amount of the sulfate; a linear response was obtained in the range of 0.5—4 μg/ml.

Sulfuric Acid Transfer between Catechol Estrogens——The assay medium (3 ml) contained catechol estrogen monosulfate (100 nmol in 0.1 ml of H_2O), catechol estrogen (100 nmol in 0.1 ml of MeOH), PAP (200 nmol in 1 ml of 1 mM Tris-HCl buffer (pH 8.7)), enzyme preparation (0.5 ml of 105000 g supernatant: 3 mg protein) and 1.3 ml of 0.1 M phosphate buffer (pH 5.0). The mixture was incubated and processed in the same manner as described above.

Assay Procedure for Enzymic Glucuronidation——The microsome fraction of guinea pig liver homogenate was used for enzymic glucuronidation. The enzymic activity was measured according to the procedure described in the previous paper.[2]

Results and Discussion

Initially, catechol estrogen monosulfates formed by incubation were unequivocally characterized by means of HPLC/ECD. The peak area ratio of the isomeric monosulfates on the chromatogram was not changed, even when acetonitrile–0.5% ammonium dihydrogenphosphate (1 : 3) was used as the mobile phase. Reduction of the 17-ketone with sodium borohydride under ice-cooling provided the corresponding 17β-hydroxyl compound. Upon usual solvolysis, catechol estrogen sulfates disappeared on the chromatogram. Known amounts of catechol estrogen monosulfates were added to the standard incubation mixture

TABLE I. Recovery of Catechol Estrogen Monosulfates Added to the Incubation Mixture[a]

Compound	Added amount	
	1 μg/ml	4 μg/ml
2-Hydroxyestrone 2-sulfate	0.810 (81.0 ± 4.0)[b]	3.476 (86.9 ± 3.9)
2-Hydroxyestrone 3-sulfate	0.777 (77.7 ± 3.7)	3.500 (87.5 ± 2.0)
2-Hydroxyestradiol 2-sulfate	0.741 (74.1 ± 2.9)	—
2-Hydroxyestradiol 3-sulfate	0.861 (86.1 ± 3.7)	—
4-Hydroxyestrone 3-sulfate	0.956 (95.6 ± 4.0)	3.364 (84.1 ± 3.0)
4-Hydroxyestrone 4-sulfate	0.940 (94.0 ± 4.3)	3.616 (90.4 ± 2.1)
4-Hydroxyestradiol 3-sulfate	0.944 (94.4 ± 4.0)	—
4-Hydroxyestradiol 4-sulfate	0.853 (85.3 ± 4.0)	—
p-Dimethylaminobenzoic acid (IS)	0.840 (84.0 ± 4.0)	—

a) Results are given in μg/ml. Both guinea pig and rat liver supernatants were used.
b) Figures in parentheses represent the mean recovery ± S.D. (%). $n \geq 7$.

TABLE II. *In Vitro* Formation of Catechol Estrogen Monosulfates from 2- and 4-Hydroxyestrones by Guinea Pig Liver[a)]

2-Hydroxyestrone		4-Hydroxyestrone	
2-Sulfate	3-Sulfate	3-Sulfate	4-Sulfate
(pmol/mg protein/min)			
2150	70	100	306

a) Figures represent the mean values obtained in duplicate experiments under the standard assay conditions (incubation time, 10 min).

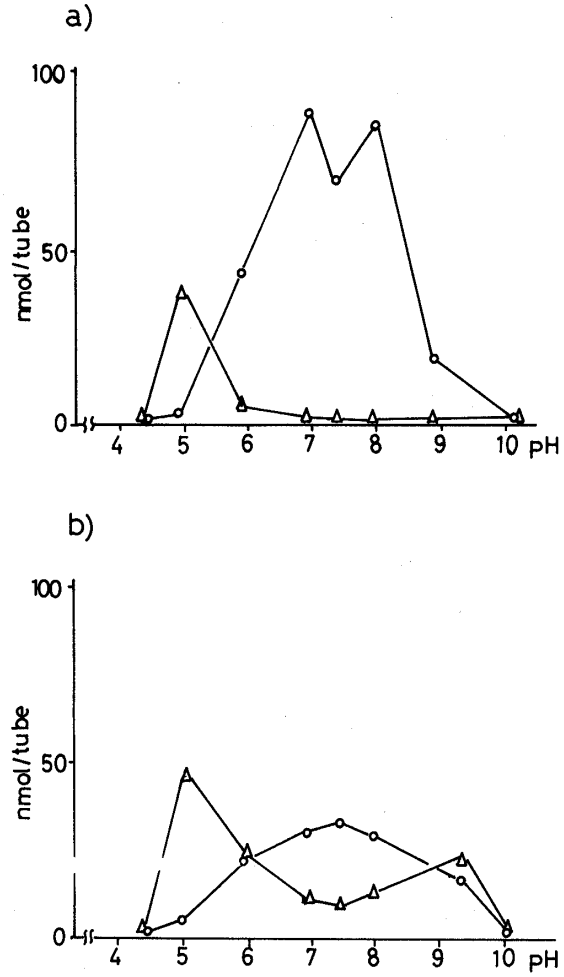

Fig. 1. Effect of pH on the Formation of Isomeric Monosulfates from Catechol Estrogens by Guinea Pig Liver

Substrate: a) 2-hydroxyestrone; b) 4-hydroxyestrone.
Product: a) ○—○, 2-hydroxyestrone 2-sulfate; △—△, 2-hydroxyestrone 3-sulfate; b) ○—○, 4-hydroxyestrone 4-sulfate; △—△, 4-hydroxyestrone 3-sulfate. Each point represents the mean value obtained in duplicate experiments.

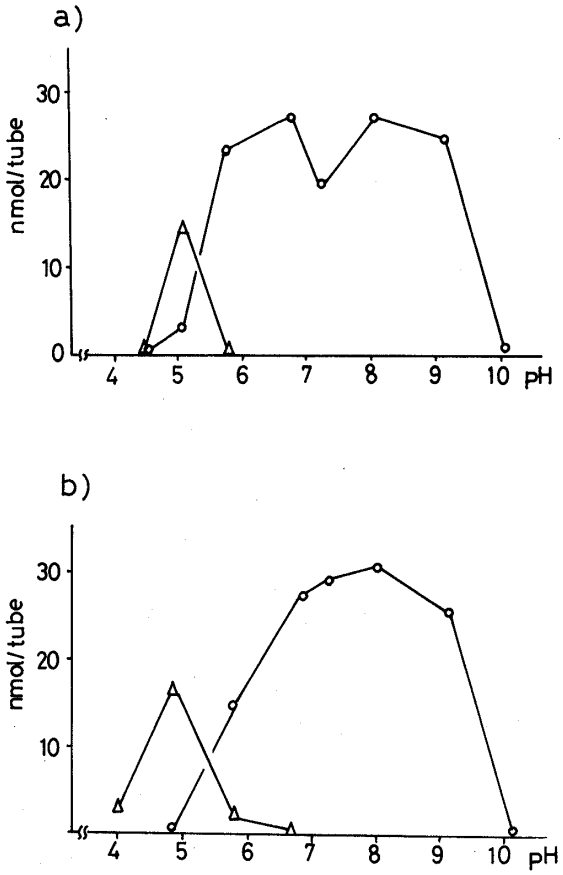

Fig. 2. Effect of pH on the Formation of Isomeric Monosulfates from Catechol Estrogens by Rat Liver

The substrates and products were the same as in Fig. 1.

containing the denatured enzyme, and their recovery rates were estimated. Each catechol estrogen monosulfate (spiked at two levels, 1.0 and 4.0 μg/ml) was recovered at a rate of more than 74% (Table I).

The subcellular distribution of the enzyme involved in the sulfation of catechol estrogens was investigated with guinea pig liver. The enzymic activity was found to be localized in the cytosol fraction. The Lineweaver–Burk plot under neutral conditions (pH 7.4) gave apparent K_m values of 11.3 and 5.3 μM for 2- and 4-hydroxyestrones, respectively. Incubation of 2- and 4-hydroxyestrones with the enzyme preparation gave the 2- and 4-sulfates, respectively, as the main product together with the 3-sulfate (Table II). It has previously been demonstrated that the rat liver cytosol fraction is capable of forming the 2- or 4-sulfate as a sole product from isomeric catechol estrogens.[2)]

The effect of the pH of the incubation medium on enzymic sulfation was then examined with guinea pig liver cytosol. The optimal formation of the monosulfates from isomeric catechol estrogens was observed at three different pHs. In the lower pH region (near 5.0) the 3-sulfate was produced, while in the neutral region (pH 7—8) the 2- or 4-sulfate was formed. In the higher pH region (near 9.4), however, the 3-sulfate was produced from 4-hydroxyestrone (Fig. 1a, b). This result prompted us to examine the effect of pH on the sulfation of the two catechols by rat liver. Incubation of the substrate with the cytosol fraction followed by analysis of the products was similarly carried out. As illustrated in Fig. 2a, b, the 3-sulfate was formed in the lower pH region while the 2- or 4-sulfate was produced in the neutral pH region. The formation of the 3-sulfate was not observed in the higher pH region.

When the substrate was incubated with the denatured enzyme preparation under the conditions described above, no sulfated product was formed. Recently, Duffel *et al.* demonstrated that sulfuric acid transfer between two phenols was catalyzed by aryl sulfotransferase in the presence of PAP.[10)] As regards catechol estrogens, however, no evidence was obtained for the occurrence of sulfuric acid transfer between the phenolic groups.

The pH-dependency of enzymic glucuronidation was also investigated for catechol estrogens. The formation of the isomeric glucuronides with liver microsomes from guinea pig was unaffected by pH in the range of 5.0—10.0.

Although the effect of pH on enzymic O-methylation of 2-hydroxyestrogens has previously been demonstrated,[11,12)] this is the first report showing that the product ratio of the isomeric monosulfates formed from the catechol is dependent upon the pH of the incubation medium. The pH-dependent formation of the isomeric catechol estrogen monosulfates might be due to alteration in the molecular species of the substrate, multiplicity of sulfotransferase, or conformational change of the enzyme. The first explanation appears to be incompatible with the virtually indistinguishable chemistry of the two phenolic groups of 2-hydroxyestrogens.[13)] No plausible explanation is at present available for the variability of enzymic sulfation. Purification of the enzymes involved in sulfation is clearly essential. Further studies on the conjugation of catechol estrogens are being conducted in these laboratories, and the details will be reported in the near future.

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