IN VITRO O'-METHYLATION OF 4-HYDROXYESTRONE MONOSULFATES

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In vitro O'-methylation of 4-hydroxyestrone monosulfates has been examined by means of high-performance liquid chromatography with electrochemical detection. When 4-hydroxyestrone or its 3-sulfate was incubated with rat liver homogenate in the presence of S'-adenosyl-L-methionine, the 4-methyl ether was formed in twelve times larger amount than the 3-methyl ether. 4-Hydroxyestrone 4-sulfate served for O'-methylation to much less extent as a substrate namely, only a small amount of the 4-methyl ether was formed. Enzymic sulfation of 4-hydroxyestrone with rat liver 105000 g supernatant fortified with adenosine 3'-phosphate 5'-phosphosulfate provided solely catecholestrogen 4-sulfates. The participation of catechol O'-methyltransferase and aryl sulfatase in the formation of guaiacol estrogens has been discussed.

Keywords— 4-hydroxyestrone; 4-hydroxyestrone monosulfate; catechol O'-methyltransferase; sulfation; guaiacol estrogen; high-performance liquid chromatography; electrochemical detection

The occurrence of 4-hydroxyestrogens besides well-known 2-hydroxyestrogens in pregnancy urine has recently been demonstrated by means of high-performance liquid chromatography (HPLC) with electrochemical detection (EDD). It is an attractive problem to clarify the metabolic fate of these catechols in connection with their physiological role in living animals. 4-Hydroxyestrone is reported to be preferentially converted into the 4-methyl ether when incubated with catechol O'-methyltransferase (COMT). This result is of particular interest because the 4-hydroxyl group is much less active than the 3-hydroxyl one toward chemical reactions. The isomeric catechols, 2-hydroxyestrogens, are transformed in vivo mainly into the 2-methyl ether while in vitro into the 2- and 3-methyl ethers in an equal amount. This apparent conflict has been explained in terms of the participation of sulfation in selective O'-methylation of the catechol.

In these respects, it appears to be of particular interest to clarify whether metabolic conjugation is similarly involved in selective O'-methylation of 4-hydroxyestrogens. The present study has been aimed at elucidation of in vitro O'-methylation of 4-hydroxyestrone monosulfates by means of HPLC/EDD.

MATERIALS AND METHODS

Chemicals and HPLC — All catechol and guaiacol estrogens, and adenosine 3'-phosphate 5'-phosphosulfate (PAPS) were prepared in our laboratories. Other chemicals used were commercially available. HPLC was performed on a Toyo Soda 803 A liquid chromatograph equipped with an EC 8 electrochemical detector and a TSK-410 column (5μm: 25 cm × 0.4 cm i.d.) (Toyo Soda Co., Tokyo) at room temperature. The potential was set at +0.9 V vs. Ag/AgCl reference electrode and the flow rate at 1.0 ml/min.

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Enzyme Preparation — Male Wistar rats weighing 200—300 g were used. Fresh liver was homogenized in ice-cold 0.25 M sucrose solution to bring a final concentration to 20%. The homogenate was centrifuged for 15 min at 1500 × g, and the supernatant was further centrifuged for 60 min at 105000 × g. Protein was determined by the method of Lowry et al. \(^5\) using bovine serum albumin as a reference.

Assay Procedure for Enzymic O-Methylation — The assay medium (2 ml) contained: substrate (200 nmol), S-adenosyl-L-methionine (SAM: 250 nmol), MgCl\(_2\) (5 μmol), enzyme preparation (1500 g or 105000 g supernatant: 10 mg protein), and 0.07 M phosphate buffer (pH 7.6) or 0.1 M Tris-HCl buffer (pH 7.5). The mixture was incubated under anaerobic condition at 37°C for 90 min. After addition of the internal standard (IS: 4-nitroestradiol), the incubation mixture was deproteinized with heat and centrifuged. The supernatant was separated and extracted with ethyl acetate. A portion of the extract was applied to HPLC/ECD (mobile phase: 0.5% NH\(_4\)H\(_2\)PO\(_4\)/acetonitrile (3:2)) for the determination of guaiacol estrogens. The aqueous layer was submitted to solvolysis in the manner used for the determination of guaiacol estrogen sulfates.

Assay Procedure for Enzymic Sulfation — The assay medium (16 ml) contained: substrate (700 nmol), PAPS (700 nmol), enzyme preparation (105000 g supernatant: 20 mg protein), and 0.1 M Tris-HCl buffer (pH 7.5) containing 0.1 mM dithiothreitol. The mixture was incubated in air at 37°C for 120 min. After addition of p-dimethylaminobenzoic acid (IS), the incubation mixture was deproteinized with heat and centrifuged. The supernatant was separated and percolated through an Amberlite XAD-4 column. The eluate with methanol was applied to HPLC/ECD using 0.5% NH\(_4\)H\(_2\)PO\(_4\) (pH 3.0)/tetrahydrofuran/methanol/acetonitrile (35:1:5:10) as a mobile phase.

RESULTS AND DISCUSSION

Initially, the selectivity for in vitro O-methylation of 4-hydroxyestrogens was confirmed. 4-Hydroxyestrone was incubated with 1500 × g supernatant of rat liver. The guaiacol estrogens (4-methoxyestrone, 4-methoxyestradiol, 4-hydroxyestrone 3-methyl ether, and 4-hydroxyestradiol 3-methyl ether) formed were extracted with ethyl acetate and determined by HPLC/ECD. 4-Hydroxyestrone 3-sulfate and 4-sulfate were then incubated under the same condition. The action of aryl sulfatase was assessed by estimation of catechol estrogens yielded without addition of SAM. \(^6\) The conversion and hydrolysis rates were calculated from the amount of the substrate employed. No sub-

<table>
<thead>
<tr>
<th>Substrate</th>
<th>4-Hydroxyestrone</th>
<th>4-Hydroxyestrone 3-sulfate</th>
<th>4-Hydroxyestrone 4-sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-OMe/3-OMe</td>
<td>11.7 ± 0.5*</td>
<td>12.3 ± 1.2</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>12.5 ± 1.3**</td>
<td>14.4 ± 2.9</td>
<td>n.d.</td>
</tr>
<tr>
<td>Conversion rate (%)</td>
<td>30.5 ± 4.9</td>
<td>35.6 ± 4.9</td>
<td>&lt; 0.2</td>
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<tr>
<td></td>
<td>20.3 ± 1.1</td>
<td>2.3 ± 0.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>Hydrolysis rate (%)</td>
<td>77.7 ± 3.1</td>
<td>3.2 ± 0.4</td>
<td>&lt; 0.4</td>
</tr>
</tbody>
</table>

a) Figures represent the mean ± S.E. (n=6).

* 1500× g supernatant, ** 105000× g supernatant, *** 4-Methoxyestrone was solely formed.
4-OMe: 4-methyl ethers, 3-OMe: 3-methyl ethers, n.d.: not detectable.
stantial difference in $O$-methylation was observed between the phosphate and Tris-HCl buffer solutions. The results are collected in Table I.

The amount of the 4-methyl ether formed was twelve times more than that of the 3-methyl ether when 4-hydroxyestrone or its 3-sulfate was used as a substrate. 4-Hydroxyestrone 4-sulfate served to a much less extent as a substrate for both COMT and aryl sulfatase and only a small amount of the 4-methyl ether was formed. Both isomeric monosulfates underwent $O$-methylation preferentially at the 4-hydroxy group. Extraction of the aqueous phase following acid hydrolysis provided no additional amount of guaiacol estrogens. These findings strongly indicate that 4-hydroxyestrogen sulfates may undergo no $O$-methylation with the retention of the sulfate linkage.

The incubation study was also carried out with 105000 $\times g$ supernatant of rat liver homogenate. Similar results were obtained with 4-hydroxyestrone sulfates except for the conversion rate which was less but proportional to the hydrolysis rate. Based upon these evidences it is reasonably considered that guaiacol estrogens would be derived from 4-hydroxyestrogens formed by cleavage of the sulfate bond, and sulfation would have no directive effect on $O$-methylation.

Sulfation of 4-hydroxyestrone was then examined with rat liver 105000 $\times g$ supernatant and PAPS. In consequence, 4-hydroxyestrogen 4-sulfates (conversion rate: 38%) were solely detected on the chromatogram. In addition, 4-hydroxyestrone was incubated with rat liver 1500 $\times g$ supernatant in the presence of both SAM and PAPS. The formation of catechol 4-sulfates was detected, and the amount ratio of 4- and 3-methyl ethers was identical with that obtained when only SAM was used as a cofactor. Sulfation and $O$-methylation would proceed independently each other and preferentially at the 4-hydroxyl group under the assay conditions used.

The present results imply no involvement of sulfate conjugation in $O$-methylation of 4-hydroxyestrone and are compatible with the previous findings on 3, 4-dihydroxybenzoic acid. Further studies on the metabolism of 4-hydroxyestrogens are being conducted in our laboratories, and the details will be reported in near future.

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**REFERENCES**


