Microdetermination of Catechol Estrogens by Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry Combined with Picolinyl Derivatization

Kouwa Yamashita,* Takanori Kawahata, Madoka Takahashi, and Mitsuteru Numazawa

Faculty of Pharmaceutical Sciences, Tohoku Pharmaceutical University, Sendai, JAPAN

A simultaneous method for quantification of four catechol estrogens, namely, 2-hydroxyestrone, 2-hydroxyestriadiol, 4-hydroxyestriadiol, and 4-hydroxyestradiol by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) was developed in a positive mode for analyzing a simple picolinyl derivatization. Reaction of these estrogens with picolinyl derivatization reagent resulted in the formation of di- or tri-picolyne derivatives, and the derivatives exhibited single charged protonated molecules as base peaks in a positive mode in electrospray ionization/mass spectrometry (ESI-MS). Picolinyl derivatives of these catechol estrogens provided approximately 10 times greater ESI responses in LC-ESI-MS/MS (selected reaction monitoring) compared with those of underivatized molecules in a negative mode. The use of picolinyl derivatizations, solid-phase extraction, and an 13C-labeled internal standard helped us in determining these catechol estrogens whose quantification limit was 20 pg for each sample. The present method was applied for quantifying these catechol estrogens in a biological sample.

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1. Introduction

In estrogen metabolism, hydroxylation of the aromatic A-ring of estrogens with cytochrome P450 (CYP) enzyme systems (CYP1A1, CYP1B1, and CYP3A4) followed by the formation of catechol o-quinones generates electrophilic molecules1,2 (Fig. 1). Catechol estrogens are typically methylated by catechol O-methyltransferase producing monomethyl ethers. However, non-methylated 2,3- and 3,4-catechols are transformed by various heme-containing enzymes to reactive o-quinones proposed by previous studies (Fig. 1). The difference in carcinogenic activity between 2,3-quinones and 3,4-quinones is also of interest in relation to providing different species of adducts.3,4 The liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS) has become an important technique in enhancing the detection sensitivity in ESI.5-10 Of the pyridine-carboxylate derivatives of hydroxyestrogens, picolinyl derivatives provided protonated molecules as base peaks with high intensity, indicating an enhancement of sensitivity in LC-ESI-tandem mass spectrometry (MS/MS) in different types of hydroxyestrogens15-17 into steroid molecules. This is a very important technique in liquid chromatography-electrospray ionization tandem mass spectrometry combined with picolinyl derivatization.

Among these derivatizations, dansyl derivatization11) and pyridine sulfonyl derivatization12) have been used to produce proton-affinitive derivatives of estrogens through the phenolic hydroxy group, however, these derivatizations could not be used for alcoholic hydroxy groups in steroid molecules. In our previous report, we synthesized several pyridine-carboxylate derivatives (picolinyl, 6-methyl picolinyl, nicotinate, isonicotinate, or 2-methylnicotinate, etc.) of hydroxyestrogens and examined their mass spectral properties and sensitivity toward ESI.13) Of the pyridine-carboxylate derivatives of hydroxyestrogens, picolinyl derivatives provided protonated molecules as base peaks with high intensity, indicating an enhancement of sensitivity in LC-ESI-tandem mass spectrometry (MS/MS) in different types of hydroxyestrogens into steroid molecules.15-17 The latter method was very useful for a simple preparation of wide varieties of pyridine-carboxylic acid esters using commercially available pyridine-carboxylic acids and 2-methyl-6-nitrobenzoic anhydride as a common reagent. The broad possibilities in the application of picolinyl derivatization of a steroid hydroxy group irrespective of its nature (phenolic or alcoholic) is advantageous when compared with the recently developed sulfonyl reagents. Picolinyl derivatization of...
estradiol yielded 3,17-dipicolinate, however, the derivative provided only a single charged protonated molecule as a base peak in its ESI-mass spectrum using positive mode detection.\(^{15}\)

In this study, we have applied this derivatization method for quantification of catechol estrogens that are precursors of carcinogenic \(\alpha\)-quinones in biological systems. We have also described an application of the present method in the measurement of catechol estrogen generated from estrone and estradiol by tyrosinase.

2. Experimental

2.1 Materials and reagents

3-Hydroxyestra-1,3,5(10)-trien-17-one (estrone: E\(_1\), 1), estra-1,3,5(10)-triene-3,17\(\beta\)-diol (estradiol: E\(_2\), 2), and tyrosinase (Mushroom, 5,500 U/mg protein) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,3-Dihydroxyestra-1,3,5(10)-trien-17-one (2-hydroxyestrone: 2-OH-E\(_1\), 3a), estra-1,3,5(10)-triene-2,3,17\(\beta\)-triol (2-hydroxyestradiol: 2-OH-E\(_2\), 4a), 3,4-dihydroxyestra-1,3,5(10)-trien-17-one (4-hydroxyestrone: 4-OH-E\(_1\), 5a), estra-1,3,5(10)-triene-3,4,17\(\beta\)-triol (4-hydroxyestradiol: 4-OH-E\(_2\), 6a) were obtained from Steraloids (Newport, RI, USA). \([\text{1,2,3,4-}^{13}\text{C}_4]\)-Estrone and \([\text{1,2,3,4-}^{13}\text{C}_4]\)-estradiol \((\text{13C}_4\)-E\(_1\)) and \([\text{1,2,3,4-}^{13}\text{C}_4]\)-estradiol \((\text{13C}_4\)-E\(_2\)) were obtained from Hayashi Pure Chemical Industry (Osaka, Japan). Picolinic acid, 2-methyl-6-nitrobenzoic anhydride, and 4-dimethylaminoypyridine were obtained from Tokyo Chemical Industry (Tokyo, Japan). Liquid chromatography-mass spectrometry (LC-MS) grade methanol (MeOH), acetonitrile (MeCN), acetic acid (AcOH), and ultra-pure water were purchased from Wako Pure Chemical Industry (Osaka, Japan).

Immobilized 2-iodoxybenzoic acid (IBX-polystyrene beads) was obtained from Nova Chemicals (Calgary, Canada).

2.2 LC-ESI-MS/MS

LC-ESI-MS/MS was run on a Finnigan TSQ Quantum triple-stage quadrupole mass spectrometer (Thermo Electron, San Jose, CA, USA) that is equipped with an ESI ion source, a Surveyor auto-sampler, and an MS-pump (Thermo Electron). The column was an X-Bridge, \((100 \text{ mm} \times 2.1 \text{ mm i.d., 5 \(\mu\text{m}\), Waters}) and it was used at an ambient temperature. The mobile phase consisting of MeCN–0.1% AcOH (1:1, v/v) was used at a flow rate of 0.2 mL/min; elution was performed in an isocratic mode. ESI-MS conditions were as follows: spray voltage, 4,500 V; sheath gas nitrogen, 25 arbitrary units (gas pressure); auxiliary gas nitrogen, 15 arbitrary units (gas pressure); ion transfer capillary temperature, 350 °C; collision gas argon, 1.5 m Torr (gas pressure); ion polarity, positive. In selected reaction monitoring (SRM), an optimized collision energy and characteristic product ion were selected for each of the picolinyl derivative from the breakdown curves of the precursor ion.

2.3 Preparation of internal standards

\([\text{13C}_4]E\(_1\) (10 \(\mu\)g) was dissolved in \(N,N\)-dimethylformamide (100 \(\mu\)L). IBX-polystyrene beads (200 \(\mu\)g) were added to this solution; the resulting mixture was allowed to stand at room temperature for 30 min. The reaction mixture was filtered; then, the filtrate was diluted using 0.5 M ascorbic acid (500 \(\mu\)L). Then, it was transferred onto an Oasis HLB cartridge (1 mL–30 mg, preconditioned with MeOH (1 mL) and H\(_2\)O (1 mL)). The cartridge was washed with 15% MeCN, and it was then eluted with 90% MeCN containing 1% AcOH (v/v) to produce a mixture of \([\text{13C}_4]2\text{-OH-E}_1\) (7a) and \([\text{13C}_4]2\text{-OH-E}_1\).
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4-OH-E1 (9a) (2.5 and 3.1 μg/mL, LC-MS assay). In the same way, the mixture of [13C4]-2-OH-E2 (8a) and [13C4]-4-OH-E2 (10a) (2.8 and 2.6 μg/mL, LC-MS assay) was obtained from [13C4]-E2 (10 μg). Without further purification, these 13C-variants for each catechol estrogen were used as internal standards (IS).

2.4 Derivatization of catechol estrogens in a microscale

A mixed solution of reagents (50 μL) (picolinic acid (30 mg), 2-methyl-6-nitrobenzoic anhydride (50 mg), 4-dimethylaminopyridine (10 mg) in dry THF (1 mL)) was added to a solution of a steroid mixture (3a-6a, 100 ng) in dry THF (50 μL). Then, triethylamine (10 μL) was added to this reaction mixture, and the resulting solution was allowed to stand at room temperature for 60 min. The resulting mixture was diluted with 5% NaHCO3 (1 mL), then, it was transferred onto an Oasis HLB cartridge (1 mL–30 mg, Waters). The cartridge was washed with 5% NaHCO3 (1 mL), distilled water, 5% HCl (1 mL), and it was again washed with distilled water (1 mL). The derivatives were eluted with 90% MeCN (1 mL) from the cartridge. After evaporating the solvent, the residue was dissolved in the mobile phase, and they were then subjected to LC-ESI-MS/MS. In a similar way, the mixture of [13C4]-2-OH-E2 (8a) and [13C4]-4-OH-E2 (10a) was transferred into the Oasis HLB cartridge. The cartridge was washed with 15% MeCN containing 1% AcOH (v/v) (1 mL); then, the steroids were eluted with 90% MeCN containing 1% AcOH (v/v) (1 mL). The eluate was evaporated to dryness in a nitrogen stream, and the residue thus obtained was dissolved in dry THF (50 μL). Then, the resulting solution was subjected to derivatization, purification, and LC-ESI-MS/MS (SRM) detection, as described in Section 2.4. Calibration curves for each steroid were obtained by assigning the concentration of 3a, 4a, 5a, or 6a to x, and the peak area ratio of picolinyl derivatives 3b, 4b, 5b, or 6b to the corresponding IS (7b, 8b, 9b, or 10b) to y, respectively. A simple linear regression was performed subsequently using the data for each steroid to construct the calibration curve.

2.6 Determination of catechol estrogens generated from estrone and estradiol by tyrosinase

To a tyrosinase solution (Mushroom) (200 μg/200 μL, 50 mM phosphate buffer, pH 7.2) was added a mixture of 1 and 2 (10 μg each/20 μL dimethylsulfoxide), and the resulting solution was incubated at 37℃ for 30 min. A portion of the mixture (100 μL) was mixed with 0.5 M ascorbic acid (100 μL), and IS solutions (7a, 8a, 9a, and 10a) of 10 ng each/50 μL MeCN. The resulting mixture was diluted with H2O (1 mL) and the diluted mixture was transferred into the Oasis HLB cartridge. After washing the cartridge with 15% MeCN containing 1% AcOH (v/v) (1 mL), estrogens were eluted with 90% MeCN containing 1% AcOH (v/v) (1 mL). The extracts were concentrated, and they were then subjected to picolinyl derivatization and analyzed by LC-ESI-MS/MS (SRM), as described earlier.

3. Results and Discussion

2-OH-E1, 2-OH-E2, 4-OH-E1, and 4-OH-E2 (3a, 4a, 5a, and 6a) were converted to the corresponding dipicolinyl or tripicolinyl derivatives using picolinic acid and 2-methyl-6-nitrobenzoic anhydride in the presence of 4-dimethylaminopyridine and triethylamine in THF solution, as described earlier5,6) (Fig. 2). Picolinyl derivatives of these four catechol estrogens were eluted in the order of 5b, 3b, 4b, and 6b, respectively, using a octadecylsilica-reversed phase column and MeCN–0.1% AcOH (1:1, v/v) as the mobile phase. Each component exhibited a well-resolved liquid chromatographic peak. LC-ESI-MS and MS/MS data of picolinyl derivatives of these four catechol estrogens are listed in Table 1.

Figure 3A shows the positive ESI-MS spectra of 3b and its [13C4]-variant (7b). ESI-MS spectra were dominated by the appearance of protonated molecules ([M+H]+) as base peaks (m/z 497.3 and m/z 501.3), which were observed in the case of other hydroxysteroids.15–17 The adduct ions of [M+Na]+ (m/z 519.3 and m/z 523.3) were also observed in their ESI-mass spectra, although in low abundance. As shown in Table 1, collision of [M+H]+ having a collision energy
of 31 eV resulted in the formation of characteristic product ions of [M+H-picolinic acid]+ at m/z 374 for 3b and 5b. Collision of [M+H]+ with high collision energy (36–44 eV) resulted in the formation of product ions (m/z 96 and 106) derived from the picolinyl moiety. Figure 3B shows the positive ESI-MS spectra of 4b along with its [13C4]-variant (8b). ESI-MS spectra were dominated by the appearance of protonated molecules ([M+H]+) as base peaks (m/z 604.3 and m/z 608.3). As seen from Fig. 3B, double charged protonated molecules ([M+2H]2+) were also observed at m/z 303, however, the intensity was low. The product ion of [M+H-picolinic acid]+ was observed at m/z 482 in 4b. Use of higher collision energy (34–42 eV) resulted in the formation of product ions (m/z 96 and 106) derived from the picolinyl moiety. Similar ESI-MS and tandem mass spectral patterns were obtained for 5b and 6b.

### Table 1. LC-ESI-MS and MS/MS Data for Picolinyl Derivatives

<table>
<thead>
<tr>
<th>Derivatives</th>
<th>LC-dataa (tR: min)</th>
<th>ESI-MS datab,c</th>
<th>Product ions: m/z (collision energy: eV)</th>
<th>MS/MS data</th>
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<tbody>
<tr>
<td>3b</td>
<td>4.22–4.26</td>
<td>497.3 (100)</td>
<td>519.2d 78 (47) 106 (36) 374 (31) 96 (44) 278 (32)</td>
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<tr>
<td>5b</td>
<td>3.83–3.87</td>
<td>497.3 (100)</td>
<td>519.2d 78 (48) 106 (34) 96 (44) 374 (31) 480 (28)</td>
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</tr>
<tr>
<td>4b</td>
<td>7.40–7.42</td>
<td>604.3 (100)</td>
<td>626.3d 78 (51) 106 (42) 482 (30) 376 (32) 96 (48)</td>
<td></td>
</tr>
<tr>
<td>6b</td>
<td>6.27–6.28</td>
<td>604.3 (100)</td>
<td>626.3d 78 (60) 482 (31) 106 (34) 376 (32) 358 (33)</td>
<td></td>
</tr>
</tbody>
</table>

a: X-Bridge 100 mm × 2.1 mm i.d. 5 μm, MeCN–0.1% AcOH = 1 : 1 (v/v, 0.2 mL/min)
b: m/z (intensities)
c: Spray voltage 4,500 V
d: [M+Na]+
e: SRM transition for quantification

Fig. 3. LC-ESI-mass spectra of the picolinyl derivatives of 2-hydroxyestrone (3b) and its [13C4]-variant (A), and 2-hydroxyestradiol (4b) and its [13C4]-variant (B) in a positive mode. Spray voltage: 4,500 V.
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In SRM, transitions of protonated molecule to m/z 106 were used for highly sensitive detection of these compounds and to achieve better signal-to-noise ratio. Figure 4 shows typical SRM chromatograms of picolinyl derivatives of four catechol estrogens monitoring their characteristic transitions ([M+H]⁺→m/z 106). Picolinyl derivatives for each catechol estrogen provided approximately 10 times higher ESI response (judging from their signal-to-noise ratio) in their LC-ESI-MS/MS compared with those of underivatized molecules by LC-ESI-MS/MS (SRM) in a negative mode.

Calibration curves were constructed for each catechol estrogen (3a, 4a, 5a, or 6a) after picolinyl derivatization using their [13C4]-variants (7a, 8a, 9a, or 10a) as IS. Parameters of calibration curves for 3a, 4a, 5a, and 6a are listed in Table 2. Each calibration curve, as determined by simple linear regression analysis, exhibits excellent linearity in the weight range 20 to 2,000 pg/sample with a regression coefficient greater than 0.997 and small y-intercepts. The quantification limit was estimated to be 20 pg/sample for each catechol estrogen; this result was comparable to that of the dansyl derivatization, as reported by Xu et al. 11)

The present method was applied to determine concentrations of catechol estrogens in the incubation mixture of 1 and 2 with tyrosinase, which was known to exert a complicated but interesting metabolizing activity toward these estrogens. 19) Figure 5 shows the LC-ESI-MS/MS (SRM) chromatograms of picolinyl derivatives of four catechol estrogens formed from 1 and 2 using tyrosinase. As is obvious from this figure, tyrosinase possessed CYP1A1 and/or CYP1B1-like hydroxylation activities for both 1 and 2 producing the corresponding 2-hydroxylated and 4-hydroxylated metabolites. It seems that conversion capability of tyrosinase was higher toward 1 as compared to that of 2 by quantifying the amount of hydroxylation products through SRM. An extended application of this method in the analysis of metabolic profiles of these catechol estrogens has the objective of investigating inherent carcinogenicity of 1 or 2 induced by the oxidation of tyrosinase-like enzymic activity in biological systems. This application is currently being investigated in our laboratory.

4. Conclusions

A simple, sensitive, and specific simultaneous assay method for four catechol estrogens combined with picolinyl derivatization has been developed. Picolinyl derivatization and subsequent purification by solid-phase extraction has made it possible to determine these estrogens in biological sample simultaneously with high sensitivity and selectivity by LC-ESI-MS/MS (SRM). Picolinyl derivatization would be a very useful and versatile method for conducting sensitive assay of a wide variety of endogenous hydroxysteroids, synthetic glucocorticoids, and other endogenous substances by LC-ESI-MS/MS.

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References


Keywords: Liquid chromatography-tandem mass spectrometry, Electrospray ionization, Catechol estrogens, Picolinyl derivatization