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Assay of labile estrogen *o*-quinones, potent carcinogenic molecular species, by high performance liquid chromatography–electrospray ionization tandem mass spectrometry with phenazine derivatization

Kouwa Yamashita*, Akina Masuda, Yuka Hoshino, Sachiko Komatsu, Mitsuteru Numazawa

Faculty of Pharmaceutical Sciences, Tohoku Pharmaceutical University, 4-1 Komatsushima 4-Chome, Aoba-ku, Sendai, Miyagi 981-8558, Japan

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ABSTRACT

A sensitive and selective assay method for labile estrogen *o*-quinones, estrone (E_1)-2,3-quinone (Q), E_1-3,4-Q, estradiol (E_2)-2,3-Q and E_2-3,4-Q, based on the use of phenazine (Phz) derivatization with *o*-phenylenediamine and high performance liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC–ESI-MS/MS) was described. The Phz derivatives of four estrogen *o*-quinones were purified by solid phase extraction and analyzed by HPLC–ESI-MS/MS. The protonated molecule was observed as a base peak for all Phz derivatives in their ESI-mass spectra (positive mode). In multiple reaction monitoring, the transition from $[M+H]^+$ to *m*/*z* 231 was chosen for quantification. Calibration curves for the *o*-quinones were obtained using standard catechol estrogens after sodium metaperiodate treatment and Phz derivatization. Using this method, these four estrogen *o*-quinones were analyzed with the limit of quantification of 5 ng/ml in acetonitrile (MeCN)–blank matrix (1:4, v/v), respectively, on a basis of the weight of catechol estrogens. Assay accuracy and precision for four estrogen *o*-quinones were 89.6–113.0% and 3.1–12.6% (5, 125 and 2000 ng/ml in MeCN–blank matrix). Applications of this method enabled to determine the catalytic activities on hydroxylation and subsequent oxidation of E_1 and E_2 of Mushroom tyrosinase and rat liver microsomal fraction. It was confirmed by this method that tyrosinase exhibited 2- and 4-hydroxylation and further oxidation activities for catechols in the ring-A of estrogens. Whereas rat liver microsomal fraction possessed only 2- and 4-hydroxylation activities, and further oxidation activity for catechol estrogens was low.

1. Introduction

It is known that estrogens exert inherent carcinogenic activities by generating electrophilic molecular species such as *o*-quinones or semiquinones that can covalently bind to DNA in addition to its hormonal action to stimulate proliferation in receptor-mediated cancer initiation [1–4]. In estrogen metabolism, hydroxylation of aromatic A-ring with CYPs enzymes and subsequent oxidation of catechols to the corresponding *o*-quinones have been hypothesized to be leading cause of generation of electrophilic molecules [5,6] (Fig. 1). Catechol estrogens are typically methylated by catechol O-methyl-transferase to afford monomethyl ethers, however non-methylated 2,3- and 3,4-catechols are transformed with peroxidase or tyrosinase to the reactive *o*-quinones that are proposed to attack nucleophilic groups on DNA via Michael addition [7–9]. It was also demonstrated that tyrosinase was responsible for the direct transformation of estrogens to *o*-quinone and further oxidized products [10]. 2,3-Quinones and 3,4-quinones provided different species of adducts [4,9,11], and these facts were another interest in relation to the existence of the difference in carcinogenic activity between these *o*-quinones.

Most of studies in this field were focused on the investigation with the purpose of obtaining insight into the possible mode of binding mechanism of such *o*-quinones to DNA or glutathione by analyzing the formed adduct qualitatively [3,4,7,12–16]. Synthesis and characterization of estrogen *o*-quinones in a preparative scale was investigated by Abul-Hajji and Dwivedy et al using ^{1}H nuclear magnetic resonance (NMR) spectroscopy and high performance liquid chromatography (HPLC) with ultraviolet (UV) detection [3,17]. In those reports, it was demonstrated that 3,4-quinones were more stable than 2,3-quinones by the measurement of their half-lives in the solution. Gelbke and Knuppen demonstrated the specific conversion of 2-hydroxyestrogens to the corresponding 2,3-quinones under various conditions and its stability, and investigated further transformation to phenazine (Phz) derivatives to stabilize *o*-quinones [18]. And it was also confirmed that prepared estrogen 2,3-quinone was relatively unstable and not obtained

* Corresponding author. Tel.: +81 22 727 0079; fax: +81 22 727 0137.
E-mail address: kyama@tohoku-pharm.ac.jp (K. Yamashita).

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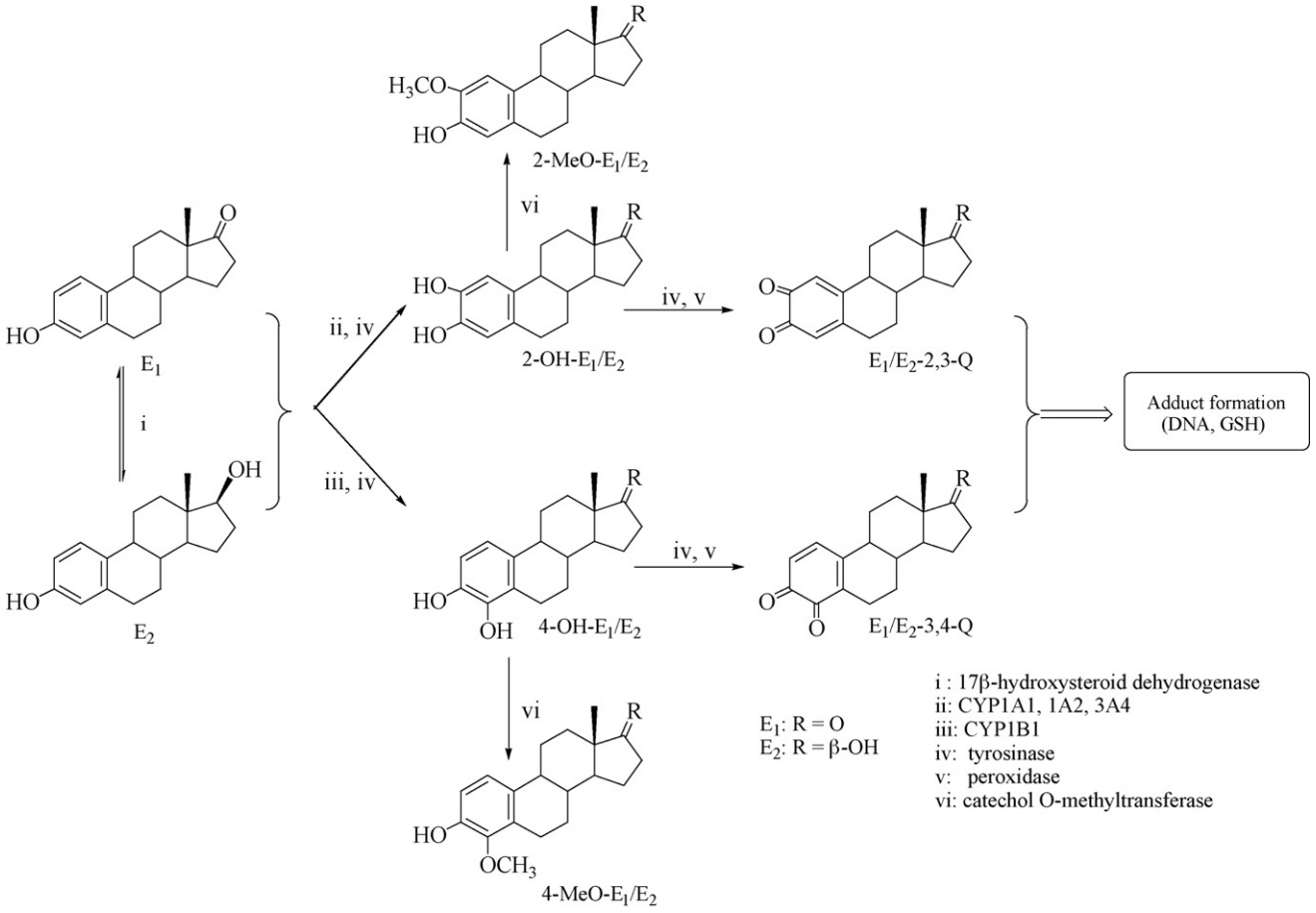

Fig. 1. Possible metabolic pathway of estrone and estradiol with various enzymic systems involving *o*-quinone formation E_1 (estrone), E_2 (estradiol), 2-OH-E_1/E_2 (2-hydroxyestrone/estradiol), 4-OH-E_1/E_2 (4-hydroxyestrone/estradiol), 2-MeO-E_1/E_2 (2-methoxyestrone/estradiol), 4-OH-E_1/E_2 (4-methoxyestrone/estradiol), E_1/E_2-2, 3-Q (estrone/estradiol-2,3-quinones) and E_1/E_2-3,4-Q (estrone/estradiol-3,4-quinones).

by crystalline form, however 2,3-quinones survive in chloroform solution for several hours [18]. The Phz derivatization was also used to the identification of 2-hydroxyestrone in human urine after oxidation [19] and other natural product [20]. However, the feasibility and selectivity of this derivatization reaction in the discriminative determination between *o*-quinone and hydroquinone, especially in a microscale, have been unclear. In this report, we described an assay method for the selective quantification of estrogen *o*-quinones in a microscale based on the use of high performance liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC–ESI-MS/MS) involving *in situ* generation of *o*-quinones from catechols and its trapping by derivatization with *o*-phenylenediamine. A Phz derivative of deuterated estrogen was synthesized and used as an internal standard for quantification. The present method was applied to the determination of the catalytic activities of Mushroom tyrosinase and rat liver microsomal fraction in the conversion of estrone and estradiol to their corresponding *o*-quinones.

2. Experimental

2.1. Materials and reagents

3-Hydroxyestra-1,3,5 (10)-trien-17-one (E_1: estrone, **1**), estra-1,3,5(10)-triene-3,17β-diol (E_2: estradiol, **2a**), estra-1,3,5(10)-triene-3,17β-diol 17-acetate (E_2-17-OAc: estradiol 17-acetate, **2b**), Mushroom tyrosinase (EC 1.18.14.1, 5500 U/mg protein) and β-nicotineamide adenine dinucleotide phosphate (reduced form) (NADPH) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 2,3-Dihydroxyestra-1,3,5(10)-trien-17-one (2-OH-E_1: 2-hydroxyestrone, **3**), estra-1,3,5(10)-triene-2,3,17β-triol (2-OH-E_2: 2-hydroxyestradiol, **4**), 3,4-dihydroxy-estra-1,3,5(10)-trien-17-one (4-OH-E_1: 4-hydroxyestrone, **5**), estra-1,3,5(10)-triene-3,4,17β-triol (4-OH-E_2: 4-hydroxyestradiol, **6**) were obtained from Steraloids (Newport, RI, USA). *o*-Phenylenediamine, sodium metaperiodate ($NaIO_4$), N, N-dimethylformamide (DMF), dimethylsulfoxide (DMSO), 36% hydrochloric acid (HCl), ethyl acetate (EtOAc), hexane, 95% ethanol (EtOH) and $NaIO_4$ were obtained from Nacalai Tesque (Kyoto, Japan). These solvents and reagents were of analytical grade. HPLC grade methanol (MeOH), acetonitrile (MeCN), acetic acid (AcOH) and ultra-pure water were purchased from Wako (Osaka, Japan). Immobilized iodoxybenzoic acid (IBX-polystyrene beads) was obtained from Nova Chemicals (Calgary, Canada). Bond Elut C_{18} (200 mg, 3 ml) and Bond Elut SI (100 mg, 1 ml) cartridges were obtained from Varian (Palo Alto, CA, USA). Pre-coated plate for thin layer chromatography (TLC) (Kiesel Gel 60 F254, 20 cm × 20 cm, 0.5 mm thickness) was obtained from Merck KGaA (Darmstadt, Germany). Freshly prepared solutions of catechol estrogens were used for each experiment. Rat liver microsomal fraction (Four weeks old, male, Wistar strain, 20 mg protein/ml) used in this study was prepared [21] and donated by Dr. Yorihisa Tanaka (Department of Drug Metabolism and Pharmacokinetics, Tohoku Pharmaceutical University).