Journal of Steroid Biochemistry & Molecular Biology 119 (2010) 141–148

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

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

ARTICLE INFO

Article history:
Received 7 October 2009
Received in revised form 12 February 2010
Accepted 18 February 2010

Keywords:
Liquid chromatography–electrospray ionization tandem mass spectrometry
o-Quinone
Immobilized iodoxybenzoic acid
Phenazine derivative
Tyrosinase
Rat liver microsomes

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).

0960-0760/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2010.02.016

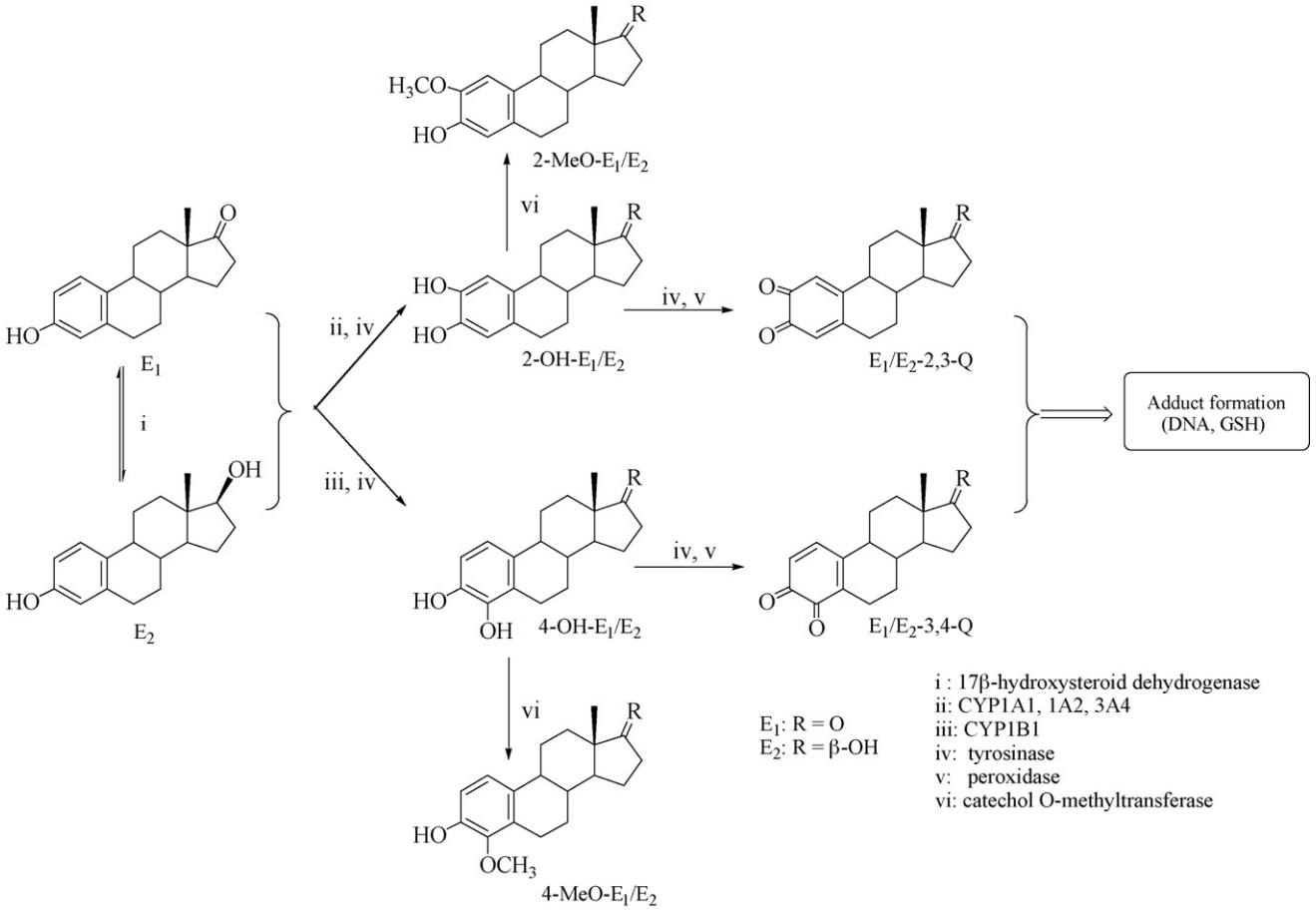

Fig. 1. Possible metabolic pathway of estrone and estradiol with various enzymic systems involving *o*-quinone formation E_1 (estrone), E_2 (estradiol), 2-OH-E_1/E_2 (2-hydroxyestrone/estradiol), 4-OH-E_1/E_2 (4-hydroxyestrone/estradiol), 2-MeO-E_1/E_2 (2-methoxyestrone/estradiol), 4-OH-E_1/E_2 (4-methoxyestrone/estradiol),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

2.2. Preparation of phenazine (Phz) derivatives (**11**, **12a**, **13**, **14a**) (Fig. 2)

Phz derivatives of four estrogen *o*-quinones, E_1-2,3-Q-Phz (**11**), E_2-2,3-Q-Phz (**12a**), E_1-3,4-Q-Phz (**13**) and E_2-3,4-Q-Phz (**14a**), were prepared from the corresponding catechol estrogens by treatment with $NaIO_4$ in DMF followed by the cyclization with *o*-phenylenediamine as described by Gelbke [18,19]. Derivatives **11** and **13** were also prepared by the direct oxidation of **1** with immobilized IBX-polystyrene beads [22–24] followed by the treatment of crude *o*-quinones with DMF solution of *o*-phenylenediamine. Derivatives **12a** and **14a** were prepared from **2b** in the similar manner as **11** and **13**. In the preparation of these derivatives, **12b** and **14b** were initially prepared as intermediates from **2b**. Treatment of **12b** or **14b** with 5% KOH/MeOH gave **12a** or **14a**. Each Phz derivative was purified by preparative TLC using EtOAc–hexane (1:2, v/v) as a developing solvent. Structures of each Phz derivative were confirmed by the measurement of ^{1}H NMR spectra (JEOL JNM-400 spectrometer; 400 MHz; $CDCl_3$ solution, Tokyo, Japan), UV spectra (Shimadzu MPS-2450 UV–vis spectrophotometer, Kyoto, Japan) and high resolution mass spectra (HR-MS) (JEOL JMS-700 double focusing mass spectrometer) in an electron ionization mode.

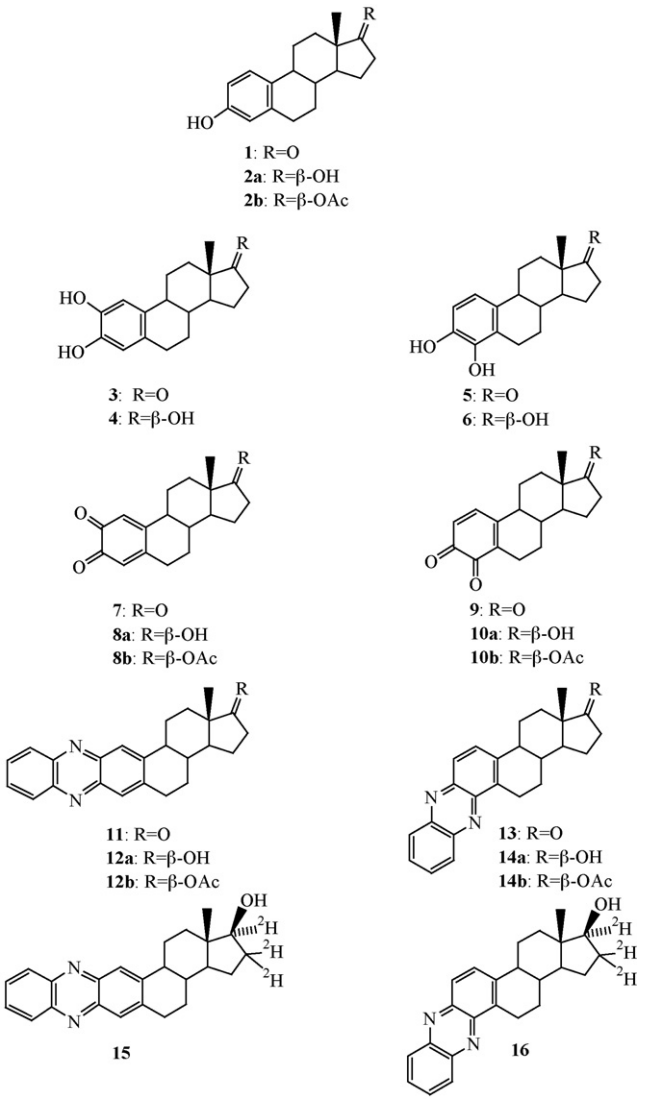

Fig. 2. Chemical structures of estrogens and phenazine derivatives used in this study.

2.3. Synthesis of internal standard (IS)

[16,16,17α-$^2H_3$] E_2-17β-OAc (15 mg) was prepared in our laboratory from [16,16,17α-$^2H_3$] E_2 by two steps of acetylation with acetic anhydride and pyridine, and partial hydrolysis of 3-acetate with $NaHCO_3$ in MeOH. [16,16,17α-$^2H_3$] E_2-17β-OAc was subsequently treated with IBX-polystyrene beads, *o*-phenylenediamine and 5% KOH in methanol to afford deuterium labeled compounds **15** (1.5 mg) and **16** (1.3 mg), after preparative TLC purification using EtOAc–hexane (1:2, v/v) as a developing solvent. Deuterium distributions were as follows: **15**: d_0; 0.5%, d_1; 3.7%, d_2; 22.5%, d_3; 70.0%, d_4; 3.3%, **16**: d_0; 0.2%, d_1; 2.8%, d_2; 22.9%, d_3; 70.9%, d_4; 3.2%. Compound **16** was used as an IS for the simultaneous determination of four estrogen *o*-quinones.

2.4. Preparation of blank matrix

Rat liver microsome suspension (20 mg protein/ml) was treated at 100 °C for 15 min. Denaturated microsome suspension was diluted with 50 mM phosphate buffer (pH 7.4, 40 ml) and then resuspended. The diluted microsome suspension was used as blank matrix.

2.5. High performance liquid chromatography (HPLC)

HPLC was run on a Waters 2695 separation module equipped with a Waters 2487 UV detector (Waters, Milford, MA, USA). The column was an X-Bridge C_{18} (150 mm × 4.6 mm I.D., 5 μm, Waters) and used at an ambient temperature. The mobile phases consisted of MeOH–H_2O–AcOH (45:55:0.1, v/v/v) for *o*-quinones or catechols and MeCN–H_2O–AcOH (70:30:0.1, v/v/v) for Phz derivatives were used at a flow rate of 1 ml/min with an isocratic elution. Wavelength of an UV detector was set at 257 nm for the detection of Phz derivatives and 280 nm for *o*-quinones and catechols, respectively.

2.6. HPLC–electrospray ionization tandem mass spectrometry (ESI-MS/MS)

HPLC–ESI-MS/MS was run on a Finnigan TSQ Quantum triple-stage quadrupole mass spectrometer (Thermo Fischer Scientific, San Jose, CA, USA) equipped with an ESI ion source and a Surveyor auto-sampler and a MS-pump (Thermo Fischer Scientific). The columns was an X-Bridge C_{18} (100 mm × 2.1 mm I.D., 5 μm, Waters) connected to a guard cartridge (C_{18}, 10 mm × 2 mm I.D., 5 μm) in a guard-cartridge holder with the mobile phase consisted of MeCN–H_2O–AcOH (70:30:0.1, v/v/v) at a flow rate of 200 μl/min. Elution was performed with an isocratic mode and at an ambient temperature. The general ESI/MS conditions were as follows: spray voltage, 4500 V (positive); sheath gas, nitrogen, 35 arbitrary unit (gas pressure); auxiliary gas, nitrogen, 15 arbitrary unit (gas pressure); ion transfer capillary temperature, 350 °C; collision gas argon, 1.5 mTorr (gas pressure). Transitions of Phz derivatives for each *o*-quinone in multiple reaction monitoring (MRM) were as follows: E_1-2,3-Q-Phz (**11**) and E_1-3,4-Q-Phz (**13**); m/z 357 → m/z 231 (collision energy, 60 V), E_2-2,3-Q-Phz (**12a**) and E_2-3,4-Q-Phz (**14a**); m/z 359 → m/z 231 (collision energy, 60 V), IS (**16**): m/z 362 → m/z 231 (collision energy, 60 V).

2.7. Generation of *o*-quinones (**7**, **9**, **8a** and **10a**) from 2- and 4-hydroxyestrogens (**3**, **5**, **4** and **6**) with $NaIO_4$ in a microscale

To a mixed solution of catechol estrogens (50 μl) (**3**, **4**, **5** and **6**: 50 μg each/ml) in MeCN–blank matrix (1:4, v/v) was added 10% $NaIO_4$ (20 μl) and AcOH (5 μl), and the resulting mixture was

allowed to stand at room temperature for 2 min. The reaction mixture was immediately analyzed by HPLC (λ 280 nm). A mixture of catechol estrogens (50 μl) (**3**, **4**, **5** and **6**) at two different concentrations (200 ng and 2000 ng/ml: MeCN–blank matrix, 1:4, v/v) were prepared and oxidized with 10% $NaIO_4$, and then submitted to LC–ESI-MS/MS analyses.

2.8. Trapping of o-quinones with o-phenylenediamine

To a solution of estrogen *o*-quinone generated as described above (Section 2.7) was added *o*-phenylenediamine (15 mg/200 μl in DMF, w/v), and the resulting mixture was allowed to stand at room temperature for 30 min. The reaction mixture was diluted 5% HCl (2 ml) and then the transferred onto Bond Elut C_{18} (200 mg, pre-conditioned with 3 ml EtOAc, 3 ml MeOH and 3 ml water). The cartridge was subsequently washed with 5% HCl (3 ml × 3), H_2O (3 ml × 3) and 35% MeCN (3 ml × 3) and the Phz derivative was eluted with EtOAc (2 ml). To this eluate was added hexane (4 ml) and the resulting mixture was passed through Bond Elut SI (100 mg, 1 ml). Eluate was evaporated to dryness and the residue was dissolved in EtOAc (100 μl) and then subjected to LC–ESI-MS/MS.

Absolute recovery rates in the processes of *o*-quinone generation from catechol estrogen followed by the Phz derivatization were assessed by comparing the peak area of the prepared Phz derivative to that of peak area obtained from the corresponding synthetic standards of each Phz derivative using **16** as an internal standard.

2.9. Calibration curves

The mixed solution of four catechol estrogens (50 μl) (**3**, **4**, **5** and **6**: 0, 2.5, 5.0, 12.5, 50, 125, 625, 2500 ng each/ml: MeCN–blank matrix, 1: 4, v/v) was subsequently treated with 10% $NaIO_4$ (20 μl) and AcOH (5 μl) at room temperature for 2 min, and then with *o*-phenylenediamine (15 mg/200 μl in DMF, v/v) at room temperature for 30 min. After addition of IS (**16**, 200 ng/20 μl MeCN), the reaction mixture was diluted 5% HCl, purified by solid phase extraction and then analyzed as mentioned above. Calibration curve for each *o*-quinone was obtained by assigning the catechol-equivalent weight to *x* and the peak area ratio of the Phz derivatives to the corresponding IS to *y*. Subsequently, a linear regression was performed for constructing calibration curve for each component.

2.10. Assay specificity

The mixed solution of four catechol estrogens (**3**, **4**, **5** and **6**: 500 ng each/50 μl: MeCN–blank matrix, 1:1, v/v) was directly treated with *o*-phenylenediamine (15 mg/200 μl DMF, w/v) and AcOH (5 μl) at room temperature for 30 min. The mixture was purified and analyzed in the similar manner as mentioned above. The peak areas corresponding to the retention time (t_R) of each Phz derivative of *o*-quinone were recorded by LC–ESI-MS/MS and compared to those of peak areas obtained for the samples by pretreatment of catechols (500 ng each) with $NaIO_4$, and then data were expressed as relative peak area.

2.11. Assay accuracy and precision

To determine the assay accuracy and precision, the mixed solution known amounts of catechol estrogens (**3**, **4**, **5** and **6**: 5, 125 and 2000 ng each/ml: MeCN–blank matrix, 1:1, v/v) was prepared and a portion of solution (50 μl) was subsequently treated with 10% $NaIO_4$ (20 μl) and AcOH (5 μl) at room temperature for 2 min, and then with *o*-phenylenediamine (15 mg/200 μl in DMF, v/v) at room temperature for 30 min. After addition of IS (200 ng/20 μl MeCN), the reaction mixture was purified as mentioned above. Within assay precision was expressed as relative standard deviation of analytical results for each concentration of sample. Accuracy was determined and expressed as a percentage of analytical as recovery rates of the measured concentrations against the spiked-concentration for each *o*-quinone (catechol estrogen-equivalent weight).

2.12. Determination of o-quinone formation from estrone and estradiol by tyrosinase and rat liver microsomes

The substrate solution (E_1 and E_2: 10 μg each in DMSO 10 μl) was added to the tyrosinase solution (500 μg in 50 mM phosphate buffer, pH 7.4) (1 ml), and the resulting mixture was incubated at 37 °C. At 30 min after starting incubation, a portion of sample (50 μl) was taken out and the sample was treated with *o*-phenylenediamine (15 mg/200 μl DMF, v/v) and AcOH (5 μl) at room temperature for 30 min. After addition of IS (**16**, 200 ng/20 μl MeCN), the reaction mixture was diluted with 5% HCl (2 ml) and the Phz derivative was extracted, purified and analyzed as mentioned above. Pre-treatment samples of incubation mixture (50 μl) with 15% $NaIO_4$ (15 μl) was prepared to estimate the formation of catechol estrogens in the medium.

Similarly, the substrate solution (E_1 and E_2: 10 μg each in DMSO 10 μl) was added to the mixture of rat liver microsomes (500 μg) and NADPH (10 μg) in 50 mM phosphate buffer (pH 7.4) (1 ml), and the resulting mixture was incubated at 37 °C. At 30 min after starting incubation, a portion of sample (50 μl) was taken out and the sample was treated and analyzed as mentioned above.

3. Results and discussion

3.1. Preparation of Phz derivatives of estrogen o-quinones

Phz derivatives of four estrogen *o*-quinones (**11**, **12a**, **13**, **14a**) were prepared as standards. *o*-Quinones have been generally obtained by oxidation of catechol estrogens using various oxidants such as activated manganese dioxide, sodium iodate or $NaIO_4$ as described by Gelke et al. [18,19]. In this study, we employed direct oxidation of E_1 or E_2 with IBX-polystyrene beads [22–24]. Treatment of E_1 with IBX-polystyrene beads in DMF solution resulted in the formation of **7** and **9** as a mixture. IBX-polystyrene beads were easily removed by filtration and *o*-quinone intermediates thus obtained were then treated with *o*-phenylenediamine in DMF solution. Preparative TLC purification of crude product gave the Phz derivatives **11** and **13** as pure materials using EtOAc–hexane (1:2, v/v) as a developing solvent.

Direct oxidation of E_2 with IBX-polystyrene beads resulted in the formation of the mixture of **8a** and **10a**. In addition to the formation of **8a** and **10a**, the production of **7** and **9** as by-products was inevitable by further oxidation at 17β-OH by this reagent showing complicated HPLC and TLC patterns. Then **12a** and **14a** were prepared using **2b** as a starting material. Oxidation of **2b** with IBX-polystyrene beads followed by the treatment with *o*-phenylenediamine resulted in the formation of **12b** and **14b**. Alkaline hydrolysis of **12b** and **14b** followed by the purification with TLC using EtOAc–hexane (1:2, v/v) as a developing solvent gave **12a** and **14a** as pure materials. ^1H NMR, HR-MS and UV spectral measurement supported the structures of **11**, **12a**, **12b**, **13**, **14a** and **14b**, respectively.

3.2. HPLC–ESI-MS/MS

In ESI-MS, the Phz derivatives of these estrogen *o*-quinones gave protonated molecule ($[M+H]^+$) as the base peaks accompanying the appearance of adduct ion of $[M+H+MeCN]^+$ in each derivative. The ESI-mass spectra and product ion spectra of the Phz

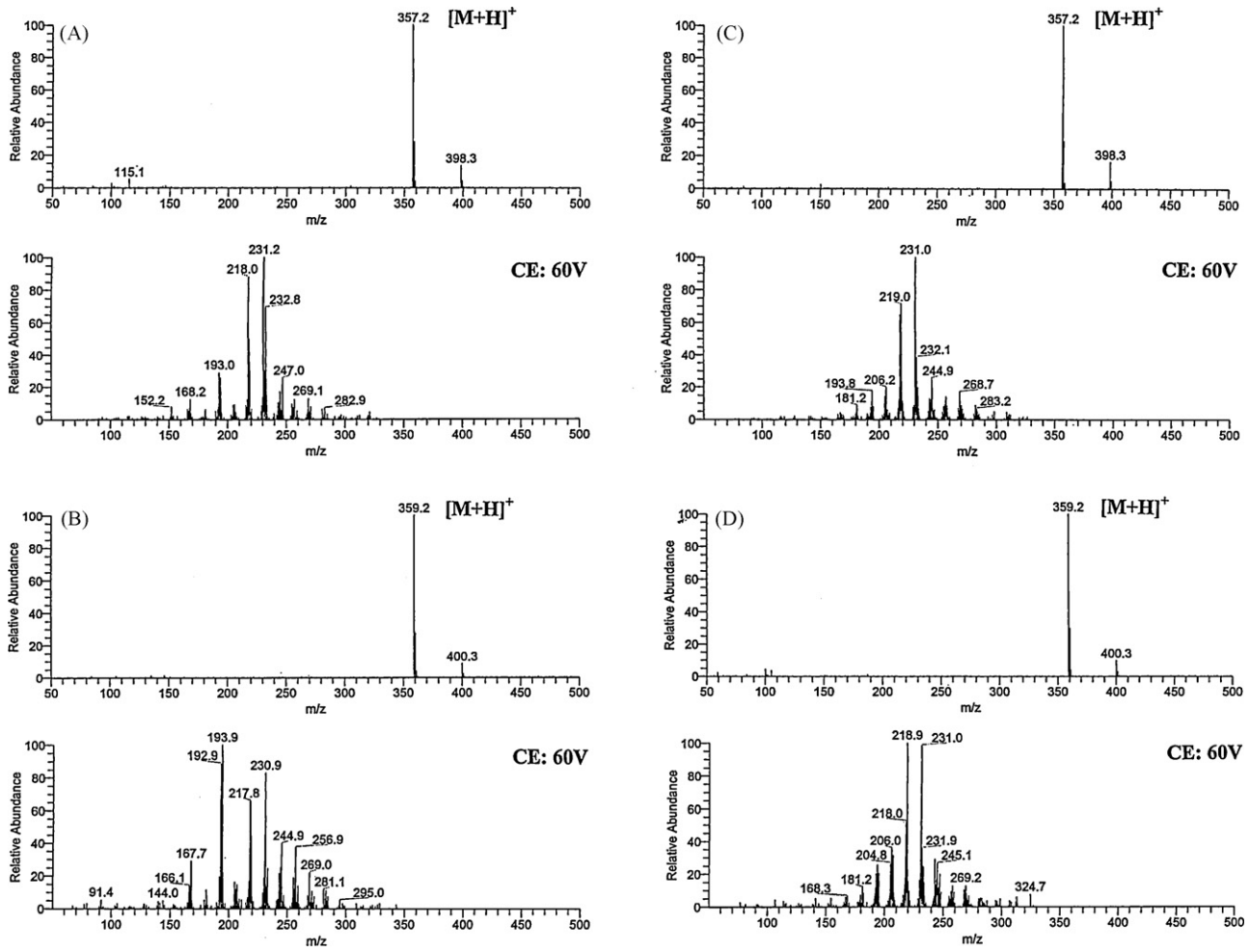

Fig. 3. Representative ESI-mass spectra (upper) and product ion mass spectra (bottom) of the Phz derivatives of estrogen *o*-quinones. (A) E_1-2,3-Q-Phz (**11**), (B) E_2-2,3-Q-Phz (**12a**), (C) E_1-3,4-Q-Phz (**13**) and (D) E_2-3,4-Q (**14a**). Spray voltage: 4500 V, polarity: positive.

derivatives E_1-2,3-Q-Phz (**11**), E_2-2,3-Q-Phz (**12a**), E_1-3,4-Q-Phz (**13**) and E_2-3,4-Q-Phz (**14a**) were shown in Fig. 3A–D. The fragmentation pattern of the base peak ion of each derivative under various levels of collision energy was examined and the ion at *m*/*z* 231 (formed by cleavage of both C_9–C_{11} and C_8–C_{14} bonds) was observed as common product ion for each Phz derivative of *o*-quinone with relatively high intensity. Then the transition from $[M+H]^+$ to *m*/*z* 231 was selected for quantification of each Phz derivative in MRM. Using reversed phase ODS-column (X-Bridge C_{18}, 100 mm × 2.1 mm I.D., 5 μm), the Phz derivative of estrogen *o*-quinones were eluted in the order **12a**, **11**, **14a** and **13**, respectively. Retention times for each of the Phz derivatives were as follows (mobile phase; MeCN–H_2O–AcOH = 70:30:0.1, v/v/v, flow rate; 0.2 ml/min): E_2-2,3-Q-Phz (**12a**, t_R = 3.4–3.5 min), E_1-2,3-Q-Phz (**11**, t_R = 3.7–3.8 min), E_2-3,4-Q-Phz (**14a**, t_R = 5.4–5.5 min) and E_1-3,4-Q-Phz (**13**, t_R = 6.0–6.1 min). All Phz derivatives of estrogen *o*-quinones were successfully separated using this mobile phase constitution.

3.3. Generation and derivatization of o-quinones in a microscale

o-Quinone generation and derivatization with *o*-phenylenediamine in a microscale was examined using catechol estrogens, **3**, **4**, **5** and **6**. In order to optimize these processes, we investigated the reaction conditions with some variations of constituents (AcOH: 1, 2, 5 and 10 μl, 10% $NaIO_4$: 10, 20 and 30 μl, *o*-phenylenediamine: 5, 10, 15, 30 mg in DMF 200 μl). The highest yields of the Phz derivatives from each catechol estrogen were obtained using the following reagent mixture; AcOH: 5 μl, 10% $NaIO_4$ 20 μl and *o*-phenylenediamine: 15 mg

Table 1
Absolute recovery rates in the processes of *o*-quinone formation and phenazine derivatization from catechol estrogens.

Concentration[a]	Absolute recovery rate[b] (%)			
	E_1-2,3-Q-Phz(**11**)	E_2-2,3-Q-Phz(**12a**)	E_1-3,4-Q-Phz(**13**)	E_2-3,4-Q-Phz(**14a**)
200 ng/ml	60.6 ± 16.2	70.7 ± 9.1	36.3 ± 8.0	55.7 ± 7.7
2000 ng/ml	66.3 ± 6.3	76.0 ± 2.9	34.5 ± 2.5	54.4 ± 0.8

Reaction conditions were as follows:
o-Quinone formation; steroids (50 μl), AcOH (5 μl), 10% $NaIO_4$ (20 μl), 2 min.
Phenazine derivatization: *o*-phenylenediamine 15 mg/200 μl DMF, 30 min.

[a] Concentrations of catechol estrogens in MeCN–blank matrix (1:4, v/v).
[b] Data were calculated on a basis of catechol estrogen-equivalent weight and expressed as the mean value of three experiments.

Table 2
Relative peak area of the generated *o*-quinones from catechols with or without $NaIO_4$ after phenazine derivatization by LC–ESI-MS/MS.

Analytes	Spiked (ng)[a]	$NaIO_4$ treatment	Relative peak area[b,c]
E_1-2,3-Q (**7**)	500	+	1.0
		–	0.0035
E_2-2,3-Q (**8a**)	500	+	1.0
		–	0.0052
E_1-3,4-Q (**9**)	500	+	1.0
		–	0.0053
E_2-3,4-Q (**10a**)	500	+	1.0
		–	0.0079

[a] Spiked amount was expressed on a basis of the weight of catechol estrogens.
[b] Peak areas were determined as their Phz derivatives.
[c] Data were presented as the mean values of three experiments.

Table 3
Assay accuracy and precision.

Analytes	Amount[a] (ng/ml)		Accuracy (%)	RSD (%)
	Added	Found (mean ± SD)[b,c]		
E_1-2,3-Q (**7**)	5	5.0 ± 0.5	100.0	10.0
	125	115.5 ± 14.5	92.4	12.6
	2000	2260.0 ± 108.5	113.0	4.8
E_2-2,3-Q (**8a**)	5	5.5 ± 0.5	110.0	9.1
	125	126.0 ± 15.1	100.8	11.9
	2000	2019.5 ± 103.5	101.0	5.1
E_1-3,4-Q (**9**)	5	5.5 ± 0.6	110.0	10.9
	125	112.0 ± 10.0	89.6	8.9
	2000	2084.0 ± 126.0	104.2	6.0
E_2-3,4-Q (**10a**)	5	5.5 ± 0.5	110.0	9.1
	125	116.5 ± 12.0	93.2	10.3
	2000	2152.5 ± 65.5	107.6	3.1

[a] Added and found values were expressed on a basis of the weight of catechol estrogens.
[b] Data were obtained from four experiments.
[c] *o*-Quinone concentrations were determined as their Phz derivatives.

in 200 μl DMF. The absolute recovery rates of each catechol estrogen were summarized in Table 1. The absolute recovery rates of **11**, **12a**, **13** and **14a** from **3**, **4**, **5** and **6** (2000 ng/ml) were 66.3 ± 6.3%, 76.0 ± 2.9%, 34.5 ± 2.5% and 54.4 ± 0.8%, respectively. Similar recovery rates were obtained for the samples with lower concentration (200 ng/ml). The absolute recovery rates in the processes with the optimized condition were varied from 35% to 76%, however, the overall yields in these processes for each catechol estrogen were reproducible in these concentrations.

3.4. Calibration curves

Calibration curves were constructed for each derivative (**11**, **13**, **12a** and **14a**) by means of catechol estrogens (**3**, **4**, **5** and **6**) after $NaIO_4$ oxidation and Phz derivatization using **16** as an internal standard. Each calibration curve, as determined by sim-

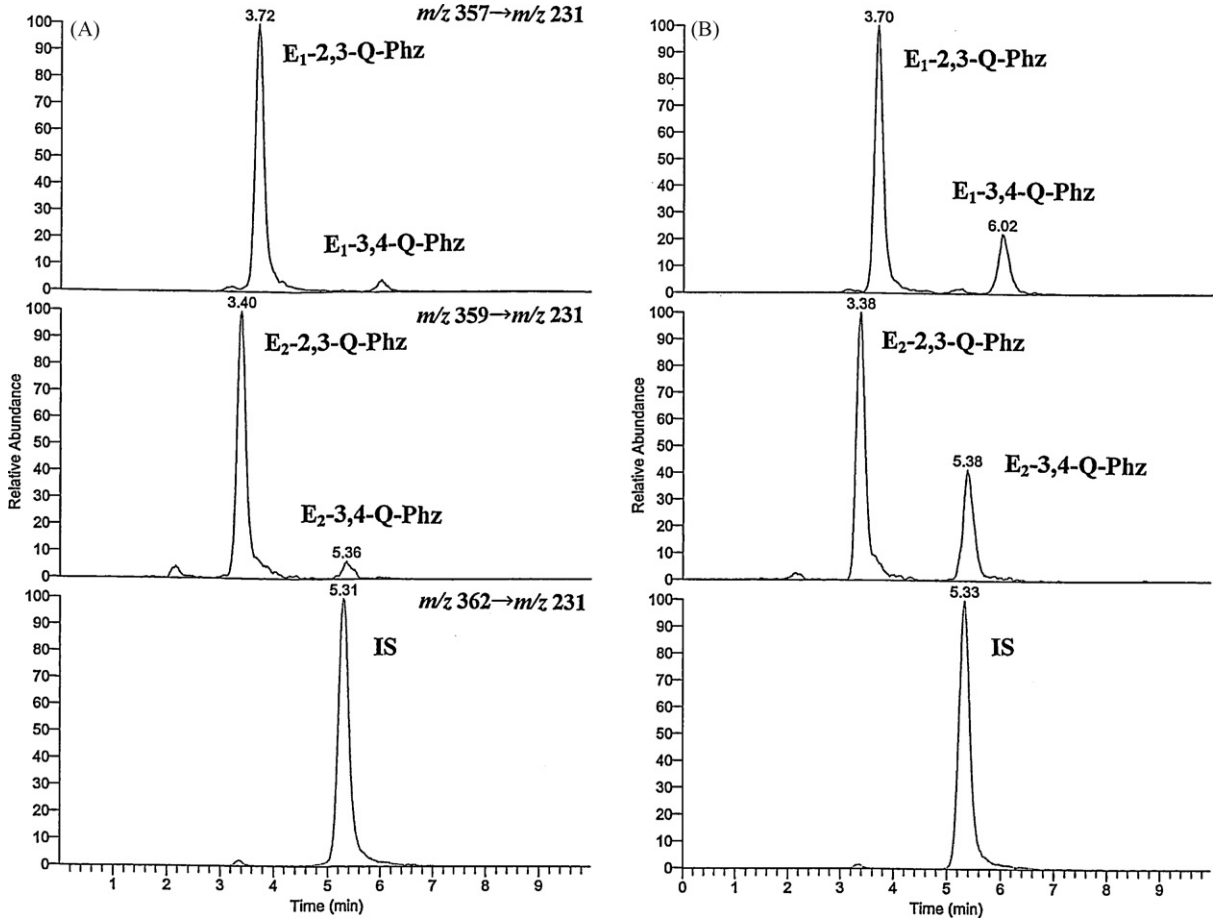

Fig. 4. Typical MRM chromatograms of *o*-quinones formed from E_1 and E_2 after incubation with tyrosinase (500 μg/ml in 50 mM phosphate buffer, pH 7.4, 37 °C for 30 min, substrate concentration: 10 μg each/ml) as their Phz derivatives. (A) Direct derivatization, (B) pre-treatment with 10% $NaIO_4$ at room temperature for 2 min. Spray voltage: 4500 V, collision energy: 60 eV. MRM transitions for each component were as follows: E_1-2,3-Q-Phz (**11**) and E_1-3,4-Q-Phz (**13**); *m*/*z* 357 → *m*/*z* 231, E_2-2,3-Q-Phz (**12a**) and E_2-3,4-Q-Phz (**14a**); *m*/*z* 359 → *m*/*z* 231 and IS (**16**): *m*/*z* 362 → *m*/*z* 231.

ple linear regression analysis, exhibited excellent linearity for the weight range of 2.5–2500 ng/ml (each catechol estrogen-equivalent weight/ml in MeCN–blank matrix, 1:4, v/v) with regression coefficient of more than 0.999 and small *y*-intercept.

3.5. Assay specificity

A degree of Phz derivatives formation from catechol estrogens (hydroquinones) with *o*-phenylenediamine without oxidation process by $NaIO_4$ was assessed by LC–ESI-MS/MS. Results were shown in Table 2. The relative peak areas corresponding to the retention times for **11**, **13**, **12a** and **14a** in the samples obtained from catechol estrogens (500 ng) were 0.0035–0.0079 when compared to those of the peak area of the same samples pre-treated with $NaIO_4$. This result suggested that the Phz derivatization was specific for estrogen *o*-quinones and enabled to determine *o*-quinones selectively even in the presence of hydroquinones.

3.6. Assay accuracy and precision

Results were given in Table 3. Assay accuracy and precision (relative standard deviation, RSD) ranged from 89.6 to 113.0% and 3.1 to 12.6%, respectively. These results indicated that the assay accuracy and reproducibility of this study were found to be satisfactory. The pure Phz derivatives (**11**, **12a**, **13**, **14a**) were detected with injected amount of 500 fg (signal-to-noise ratio of >5), however, the limit of quantifications (LOQs) for each estrogen *o*-quinone as Phz derivatives in the real sample (MeCN–blank matrix; 1:4, v/v) were estimated to be 5 ng/ml with acceptable accuracy and precision (>15%). The LOQ of this method enabled to analyze spontaneously generated estrogen *o*-quinones in the medium obtained by the incubation of E_1 and E_2 with Mushroom tyrosinase or rat liver microsomal fraction at a protein concentration of 0.5 mg/ml.

The Phz derivatives of estrogen *o*-quinones were detected by HPLC–UV method with high sensitivity (10 pg on-column, synthetic standard, data not shown) because of the large ε value of inherent Phz moiety. However, the LOQ in the real sample (MeCN–blank matrix; 1:4, v/v) was limited to be approximately 25 ng/ml due to the co-existing impurities originated from the excess derivatizing agent even after the use of solid phase purification. The typical HPLC–UV chromatogram of the Phz derivatives of generated *o*-quinones from catechol estrogens in the blank matrix was shown in Supplementary materials, which can be found in the electronic version of this article.

3.7. Catalytic activities of tyrosinase and rat liver microsomes on *o*-quinones formation from estrone and/or estradiol

Fig. 4 showed the typical MRM chromatograms of estrogen *o*-quinones formed in the medium after incubation of E_1 or E_2 with Mushroom tyrosinase after Phz derivatization. As

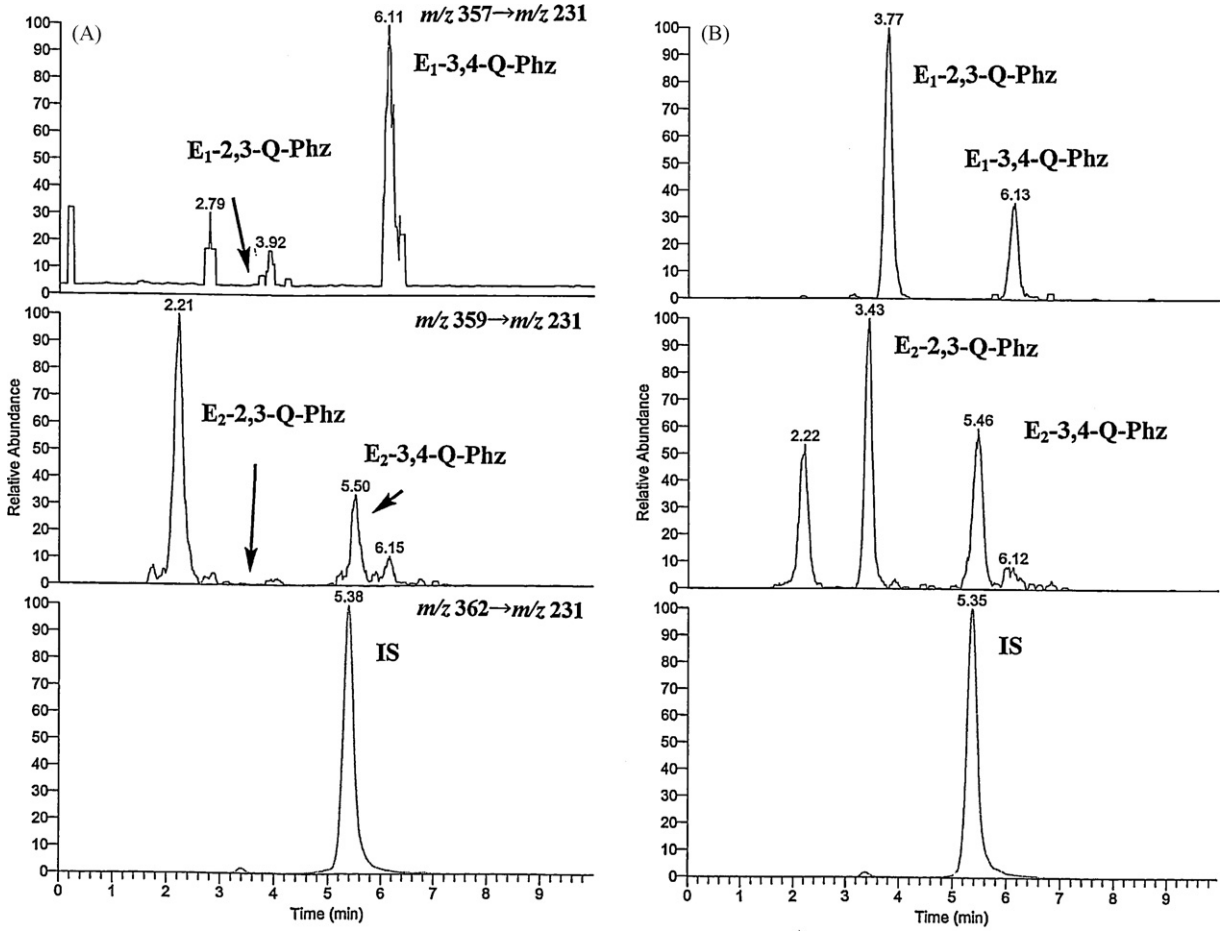

Fig. 5. Typical SRM chromatograms of *o*-quinones formed from E_1 and E_2 after incubation with rat liver microsomes (500 μg/ml in 50 mM phosphate buffer, pH 7.4, 37 °C for 30 min, substrate concentration: 10 μg each/ml) as their Phz derivatives. (A) Direct derivatization, (B) pre-treatment with 10% $NaIO_4$ at room temperature for 2 min. Spray voltage: 4500 V, collision energy: 60 eV. MRM transitions for each component were as follows: E_1-2,3-Q-Phz (**11**) and E_1-3,4-Q-Phz (**13**); *m*/*z* 357 → *m*/*z* 231, E_2-2, 3-Q-Phz (**12a**) and E_2-3, 4-Q-Phz (**14a**); *m*/*z* 359 → *m*/*z* 231 and IS (**16**): *m*/*z* 362 → *m*/*z* 231.

shown in Fig. 4A, the direct derivatization of the incubation mixture of E_1 and E_2 with tyrosinase resulted in the formation of the Phz derivatives E_1-2,3-Q-Phz (**11**) and E_2-2,3-Q-Phz (**12a**) as major products accompanying the production of E_1-3,4-Q-Phz (**13**) and E_2-3,4-Q-Phz (**14a**) in a small quantity (3.8–6.2%, w/w, of **11** and **12a**). Pre-treatment of the incubation mixture with $NaIO_4$ prior to Phz derivatization resulted in the slight increase of **11** and **12a** formation (119–137% of direct derivatization) but the tremendous increase of **13** and **14a** formations (890–913% of direct analysis) (Fig. 4B). These results indicated that tyrosinase possessed the catalytic activities for both 2- and 4-hydroxylation of the ring-A of estrogens and subsequent oxidation of catechols to 2,3-quinones. The catalytic activities of tyrosinase for 4-hydroxylation were 25–50% in comparison with those of 2-hydroxylation activities. The catalytic activity for the oxidation of 3,4-catechols to the corresponding 3,4-quinones was low in contrast to the corresponding oxidation activity for 2,3-catechols. As shown in these results, the high regio-specificities of this enzyme were observed in the oxidation processes between 2,3-catechols and 3,4-catechols to their corresponding *o*-quinones.

On the other hand, the direct derivatization of the incubation mixture of E_1 and E_2 with rat liver microsomes resulted in the detection of **11**, **12a**, **13** and **14a** with only trace levels (Fig. 5A), however the significant increases in the formations of 2,3-quinones and 3,4-quinones were observed after pre-treatment of the incubation mixture with $NaIO_4$ (Fig. 5B). These results indicated that rat liver microsomal fraction possessed mainly the catalytic activities of 2- and 4-hydroxylation of the ring-A of estrogens, which might be attributable to CYP1A1, CYP1A2, CYP3A4 and CYP1B1 activities [25], and further oxidation activities of catechols to the corresponding *o*-quinones were estimated to be low in this enzyme system. These results also suggested that rat hepatic microsomal enzymes seem not to convert primarily produced catechol estrogens to the corresponding *o*-quinones inherently.

In both enzyme systems, the difference in the specificity for hydroxylation and further oxidation of the ring-A of estrogens was not recognized between E_1 and E_2.

4. Conclusion

A sensitive and selective assay method of estrogen *o*-quinones by HPLC–ESI-MS/MS combined with the Phz derivatization was developed in a microscale. The stock solutions of standards of *o*-quinones were not available because of their instabilities, and the generation of *o*-quinones from catechol estrogens was performed *in situ* using $NaIO_4$. Generation of *o*-quinones and Phz derivatization were reproducible even in a microscale, and the Phz derivatives of *o*-quinones were sensitive toward LC–ESI-MS/MS (MRM) detection in a positive mode. Studies on the formation mechanism of such potent carcinogenic estrogen *o*-quinones in biological systems seems to be important to assess the hazard risk of endogenous/exogenous estrogens under various physiological and artificial conditions in relation to the exposures to acceleration/inhibition factors for *o*-quinone formation. The assessment of the robustness of this method and the application of this method to human serum, urine and saliva, are currently being evaluated in our laboratory.

Acknowledgement

This work was supported in part by a High Technology Research Center Project from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2010.02.016.

References

[1] J.D. Yager, N.E. Davidson, Estrogen carcinogenesis in breast cancer, N. Engl. J. Med. 354 (2006) 270–282.
[2] J.G. Liehr, Genotoxic effects of estrogens, Mutat. Res. 238 (1990) 269–276.
[3] I. Dwivedy, P. Devanesan, P. Cremonesi, E. Rogan, E. Cavalieri, Synthesis and characterization of estrogen 2,3- and 3,4-quinones. Comparison of DNA adducts formed by the quinones versus horseradish peroxidase-activated catechol estrogens, Chem. Res. Toxicol. 5 (1992) 828–833.
[4] D.E. Stack, J. Byun, M.L. Gross, E.G. Rogan, E.L. Cavalieri, Molecular characterization of catechol estrogen quinones in reactions with deoxyribonucleosides, Chem. Res. Toxicol. 9 (1996) 851–859.
[5] E.L. Cavalieri, K.-M. Li, N. Balu, M. Saeed, P. Devanesan, S. Higginbotham, J. Zao, M.L. Gross, E.G. Rogan, Catechol ortho-quinones: the electrophilic compound that form depurinating DNA adducts and could initiate cancer and other disease, Carcinogenesis 23 (2002) 1071–1077.
[6] Y. Tsuchiya, M. Nakajima, T. Yokoi, Cytochrome P450-mediated metabolism of estrogens and its regulation in human, Cancer Lett. 227 (2005) 115–124.
[7] J.L. Bolton, G.R.J. Thatcher, Potential mechanisms of estrogen quinone carcinogenesis, Chem. Res. Toxicol. 21 (2008) 93–101.
[8] E. Cavalieri, D. Chakravarti, J. Guttenplan, E. Hart, J. Ingel, R. Jankowiak, P. Muti, E. Rogan, J. Russo, R. Santen, T. Sutter, Catechol estrogen quinones as initiators of breast cancer and other human cancers: implications for biomarkers of susceptibility and cancer prevention, Biochem. Biophys. Acta 1766 (2006) 63–78.
[9] O. Convert, C.V. Aerden, L. Debrauwer, E. Rathahao, H. Molines, F. Fournier, J.-C. Tabet, A. Paris, Reaction of estradiol-2,3-quinones with deoxyribonucleosides: possible insights in the reactivity of estrogen quinones with DNA, Chem. Res. Toxicol. 15 (2002) 754–764.
[10] A. Pezzella, L. Lista, A. Napolitano, M. d'Ischia, Tyrosinase-catalyzed oxidation of 17β-estradiol: structure elucidation of the product formed beyond catechol estrogen quinones, Chem. Res. Toxicol. 18 (2005) 1413–1419.
[11] E.L. Cavalieri, D.E. Stack, P.D. Devanesane, R. Todorovic, I. Dwivedy, S. Hagginbotham, S.I. Johansson, K.D. Patil, M.L. Gross, J.K. Gooden, R. Ramanathan, R.L. Cerny, E.G. Rogan, Molecular origin of cancer: catechol estrogens-3,4-quinones as endogenous tumor initiators, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 10937–10942.
[12] E. Rathahao, A. Page, I. Jouanin, A. Parism, L. Debrauwer, Liquid chromatography coupled to negative electrospray/ion trap mass spectrometry for the identification of isomeric glutathione conjugates of catechol estrogens, Int. J. Mass Spectrom. 231 (2004) 119–129.
[13] K. Cao, D. Stack, R. Ramanathan, M.L. Gross, E.G. Rogan, E.L. Cavalieri, Synthesis and structure elucidation of estrogen quinones conjugated with cystein, N-acetylcysteine, and glutathione, Chem. Res. Toxicol. 11 (1998) 909–916.
[14] A. Akanni, K. Tabakovic, Y.J. Abul-Hajj, Estrogen-nucleic acid adducts: reaction of 3,4-estrone o-quinone with nucleic acid bases, Chem. Res. Toxicol. 10 (1997) 477–481.
[15] D.L. Hachey, S. Dawling, N. Roodi, F. Parl, Sequential action of phase I and II enzymes cytochrome P450 1B1 and glutathione S-transferase P1 in mammary estrogen metabolism, Cancer Res. 63 (2003) 8492–8499.
[16] M. Numazawa, T. Nambara, A new mechanism of in vitro formation of catechol estrogen glutathione conjugate by rat liver microsomes, J. Steroid Biochem. 8 (1977) 835–840.
[17] Y.J. Abul-Hajji, Synthesis of 3, 4-estrogen-o-quinone, J. Steroid Biochem. 21 (1984) 621–622.
[18] H.P. Gelbke, R. Knuppen, Synthesis of specific phenazine derivatives of 2-hydroxyestrogens, Steroids 21 (1973) 689–702.
[19] H.P. Gelbke, M. Kreth, R. Knuppen, A chemical method for quantitative determination of 2-hydroxyestrone in human urine, Steroids 21 (1973) 665–687.
[20] G.R. Pettit, A.J. Thornhill, B.R. Moser, F. Hogan, Antineoplastic agents. 552. Oxidation of combretastatin A-1: trapping the *o*-quinone intermediate considered the metabolic product of the corresponding phosphate prodrug, J. Nat. Prod. 71 (2008) 1561–1563.
[21] K. Itoh, M. Adachi, J. Sato, K. Shouji, K. Fukiya, K. Fujii, Y. Tanaka, Effect of selenium deficiency on aldehyde oxidase 1 in rats, Biol. Pharm. Bull. 32 (2009) 190–194.
[22] G. Sorg, A. Mengel, G. Jung, J. Rademann, Oxidizing, Polymers:, A polymer-supported recyclable hypervalent iodine (V) reagent for the efficient conversion of alcohols, carbonyl compounds, and unsaturated carbamates in solution, Angew. Chim. Int. Ed. 40 (2001) 4395–4397.
[23] M. Saeed, M. Zahid, E. Rogan, E. Cavalieri, Synthesis of the catechols of natural and synthetic estrogens by using 2-iodoxybenzoic acid (IBX) as the oxidizing agent, Steroids 70 (2005) 173–178.
[24] M. Frigerio, M. Santagostino, S. Sputore, A user-friendly entry to 2-iodoxybenzoic acid (IBX), J. Org. Chem. 64 (1999) 4537–4538.
[25] A.J. Lee, M.X. Cai, P.E. Thomas, A.H. Conney, B.T. Zhu, Characterization of the oxidative metabolites of 17β-estradiol and estrone formed by 15 selectively expressed human cytochrome P450 isoforms, Endocrinology 144 (2003) 3382–3398.