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Evaluation of electrospray ionization and atmospheric pressure chemical ionization for simultaneous detection of estrone and its metabolites using high-performance liquid chromatography/tandem mass spectrometry

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Abstract

The levels of estrogens and/or their metabolites play important roles in carcinogenesis, reproductive function, and sexual development during perinatal and adolescence periods. The main purpose of this report was to investigate the applicability of high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) with electrospray ionization (ESI) and/or atmospheric pressure chemical ionization (APCI) for simultaneous detection of estrone (E1) and its six metabolites. Both positive and negative ionization modes in ESI and APCI were used to evaluate the signal responses of seven target analytes. Among the seven target analytes, five analytes, E1, 16α-hydroxyestrone, 2-methoxyestrone, 4-methoxyestrone, and 2-hydroxyestrone-3-methyl, produced signals with the best signal-to-noise (S/N) ratios in positive APCI-MS/MS mode, while the other two analytes, 2-hydroxyestrone and 4-hydroxyestrone, yielded the best S/N ratios in negative ESI-MS/MS mode. Based on the results of the evaluation, HPLC–APCI-MS/MS with switching between positive and negative modes was recommended for simultaneous detection of E1 and its six metabolites. The proposed analytical scheme was successfully applied in the analysis of cell culture medium of Human liver carcinoma cells treated with varying amounts of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

Keywords: Estrogen; Estrone; Metabolite; Electrospray ionization; Atmospheric pressure chemical ionization

1. Introduction

The evidence that levels of estrogens play a role in the development of breast cancer is substantial [1] and the ratio of estrogen metabolites 2-hydroxyestrone (2-OH E1) to 16α-hydroxyestrone (16α-OH E1) has been suggested as an indicator for breast cancer risks [2]. Body burdens of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), an important endocrine disruptor, have been reported to associate with altered ratio of 2-hydroxyestradiol (2-OH E2) to 4-hydroxyestradiol (4-OH E2) [3]. The importance of the functional role of estrogen metabolism and the impacts of the estrogen metabolites on carcinogenesis were emphasized in many reviews [4–7]. Therefore, quantitative profiling of estrogens and their metabolites in biological fluids can provide valuable information in the fields of early diagnosis of carcinogensis and other endocrine related diseases.

Various methods have been developed for the determination of estrogens and their metabolites in biological matrices, including radioimmunoassay (RIA) [8], enzyme immunoassay (EIA) [9], gas chromatography–mass spectrometry (GC–MS) [10,11], and high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) [12–17]. The use of HPLC–MS/MS for estrogen metabolites analysis provides several advantages over current methods. Both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) combined with MS/MS have been applied for the determination of selective estrogen-related metabolites in rat brain, human urine, and human plasma [12–17].

The choice of ESI or APCI for best analytical performance is an important consideration for simultaneous detection of estrogens and estrogen metabolites using HPLC–MS/MS. The chemical properties (polarity, pK_a, etc.) of estrogens and their metabolites vary dramatically. Four comparative studies have

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been reported so far concerning the suitability of different atmospheric pressure ionization techniques, ESI and APCI, for simultaneous analysis of estrogens or their metabolites. However, three of the studies only focused on the estrogens alone, estrone (E1) and estradiol (E2) [14,18–20]. Ma and Kim reported that the sensitivities for E1 and E2 in (+)ESI-MS/MS mode are better than those in (+)APCI-MS/MS mode [18]. Lopez de Alda and Barcelo reported that the sensitivities for E1 and E2 in (−)ESI-MS/MS mode are better than those in (−)APCI-MS/MS [19]. Diaz-Cruz et al. reported that the sensitivities for E1 and E2 in (−)ESI-MS/MS mode are better than those in (+)APCI-MS/MS [20]. For the estrogen metabolites, Xu et al. reported that the sensitivities for toluenesulfonhydrazide derivatives of 2-OH E1 and 4-OH E1 in (+)ESI-MS/MS mode are better than un-derivatives of 2-OH E1 and 4-OH E1 in (+)APCI-MS/MS mode [14]. Although HPLC-MS/MS with ESI or APCI have been used for analysis of estrogens and their metabolites, the information was limited and inconsistent in terms of evaluating the relative performances of ESI and APCI sources. In this report the applicability of HPLC-MS/MS with ESI and/or APCI for simultaneous detection of E1 and its six metabolites was examined. Both positive and negative ionization modes in ESI and APCI, that is (+)ESI, (−)ESI, (+)APCI, and (−)APCI, were evaluated. Based on the results of the evaluation, an analytical procedure was recommended for simultaneous detection of E1 and its six metabolites. Furthermore, the proposed analytical scheme was successfully applied in the determination of E1 and its six metabolites in cell culture medium of Human liver carcinoma cells treated with varying amounts of 2,3,7,8-TCDD.

2. Experimental

2.1. Materials

Seven standard solutions of estrone (E1) and its metabolites, E1, 2-hydroxyestrone (2-OH E1), 4-hydroxyestrone (4-OH E1), 16α-hydroxyestrone (16α-OH E1), 2-methoxyestrone (2-OMe E1), 4-methoxyestrone (4-OMe E1), and 2-hydroxyestrone-3-methyl (2-OH-3-OMe E1), were purchased from Steraloids (Newport, RI, USA). Deuterium-labeled estrone-2,4,16,16-d_4 (E1-d_4) standard was purchased from Supelco (Bellefonte, PA, USA). The chemical structures of E1 and its metabolites are shown in Fig. 1. Methanol (HPLC grade) was purchased from J.T. Baker (Phillipsburg, NJ, USA), and acetonitrile (HPLC grade), formic acid (reagent grade), and sodium acetate (reagent grade) were from Riedel-de-Haen AG (Seelze, Germany). Dimethyl sulfoxide (DMSO), L-ascorbic acid and sodium bicarbonate were purchased from Sigma (St. Louis, MO, USA) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) was from AccuStandard (New Haven, CT, USA).

2.2. HPLC–MS/MS

An API 3000 triple-stage quadrupole mass spectrometer (PE SCIEX, Concord, Ontario, Canada) equipped with turbo electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) source was used for the determination and quantification of E1 and its metabolites by multiple-reaction-monitoring (MRM). Q1 full scan and MS/MS mass spectra of E1 and its metabolites were obtained by infusion of analyte standard solutions (1 μg/mL in 50% methanol/water containing 0.1% formic acid for positive mode and 1 μg/mL in 50% methanol/water for negative mode) at 20 μL/min into the ESI- or APCI-MS/MS. The mass spectral signals of precursor and product ions for each analyte were optimized by infusion followed by flow injection of analyte standard solutions. Voltages of ESI and APCI, emitter and corona discharge needle, respectively, were set to 5000 eV for positive mode and −3800 eV for negative mode. Both nebulizer and curtain gas were set to 12 and collision-assisted-dissociation (CAD) gas to 4 arbitrary instrumental units. Other instrumental parameters (such as orifice, ring and quadrupole voltages) were optimized for maximum signal of each individual analyte using automatic tuning function provided by the API 3000. The dwell time for MRM was set to 200 ms.

High-performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) experiments were performed using two Perkin-Elmer (Foster City, CA, USA) Series 200 micro pumps for gradient solvent delivery connected directly to the API 3000 mass spectrometer. Sample solutions were separated on a BDS Hypersil C18 column (15 cm × 4.6 mm i.d., particle size 5 μm, Thermo Waltham, MA, USA) with 40–80% gradients of acetonitrile in aqueous at a flow rate of 600 μL/min. The gradient of mobile phase started with 100% solvent A (40% acetonitrile/water, pH 4) for 15 min, ramped to 100% solvent B (80% acetonitrile/water, pH 4) within 5 min, and maintained at 100% solvent B for 5 min. The chromatographic parameters were optimized to allow the separation of the seven analytes.

2.3. Human Hep G2 cell cultures and 2,3,7,8-TCDD exposure

Human liver carcinoma cell line, Hep G2 cells (ATCC HB-8065), was maintained in a 10-cm dish containing 10 mL of Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco), 10 unit/mL penicillin G sodium (Gibco), and 1.5 mg/mL sodium bicarbonate under 5% CO_2 at 37 °C. At 70–80% confluency, the cells were treated with 10^{-6} M E1 and varying amounts of 2,3,7,8-TCDD in DMSO to yield final concentrations of 0, 0.001, 0.01, 0.1, 1.0 and 10.0 nM. At the end of the 24-h of incubation, the cell culture medium was collected and to the medium an equal volume of sodium acetate buffer (200 mM, pH 4.5) containing 0.1% L-ascorbic acid was added to stop the enzymatic reactions. The samples of cell culture medium were frozen at −20 °C immediately until the time for analysis.

2.4. Preparation of cell culture medium for LC–MS/MS analysis

The sample of cell culture medium (1.0 mL) was spiked with a deuterium-labeled internal standard, estrone-2,4,16,16-d_4 (E1-d_4) and then loaded onto a 96-well C_{18} solid-phase extraction plate (Discovery DSC-18 APE-96 plate, 100 mg/well, Supelco, Bellefonte, PA, USA) pre-conditioned sequentially with methanol (2.0 mL) and 5% methanol in water (2.0 mL).

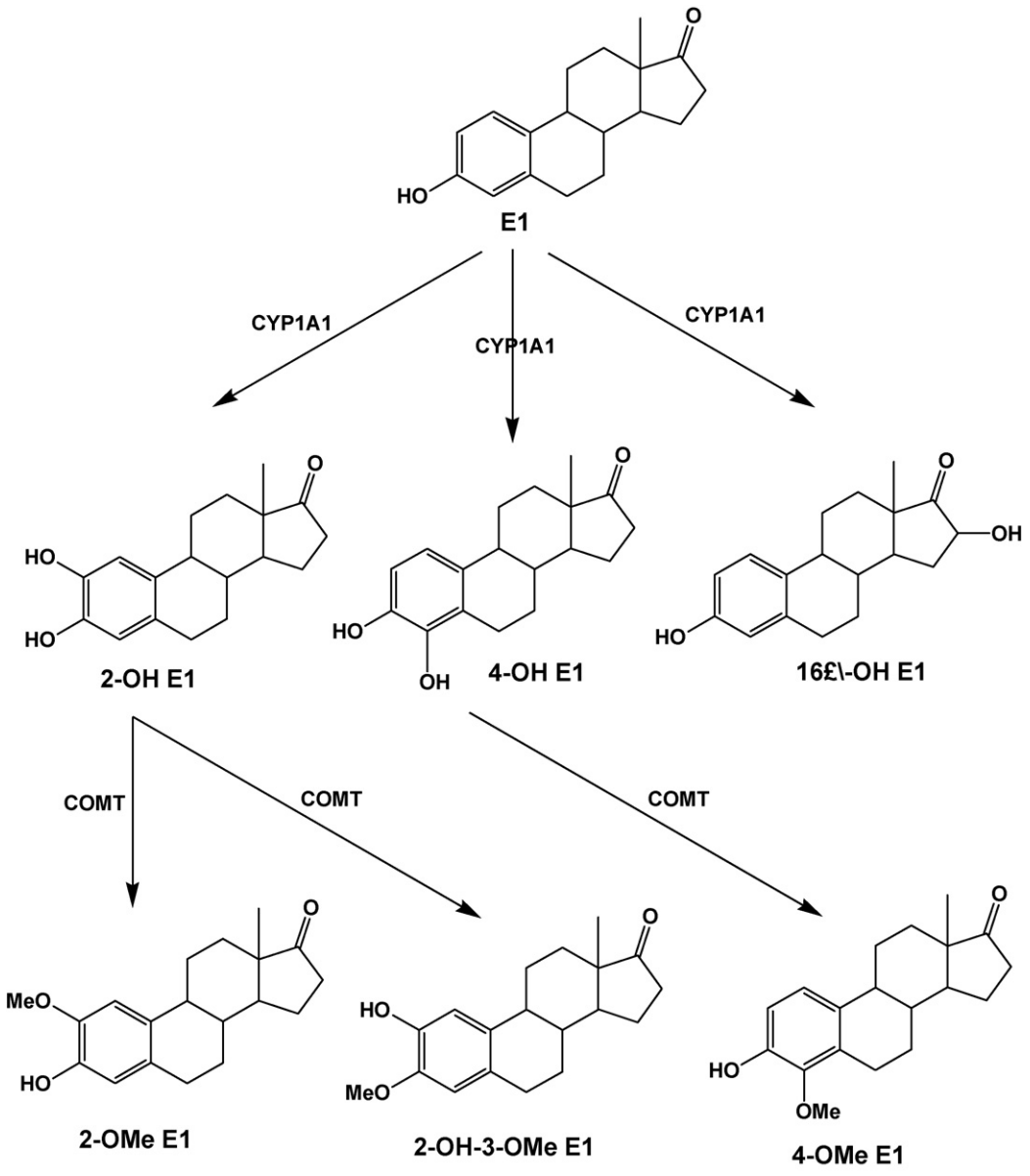

Fig. 1. Proposed formation pathway of estrone metabolites. E1: estrone, 2-OH E1: 2-hydroxyestrone, 4-OH E1: 4-hydroxyestrone, 16α-OH E1: 16α-hydroxyestrone, 2-OMe E1: 2-methoxyestrone, 4-OMe E1: 4-methoxyestrone, 2-OH-3-OMe E1: 2-hydroxyestrone-3-methyl, CYP 1A1: cytochrome P450 1A1 family, and COMT: catechol *O*-methyltransferase.

The 96-well C_{18} solid-phase extraction plate was washed with 5% methanol in water (2.0 mL × 2) followed by the elution with 100% methanol (2.0 mL) to recover the analytes. The resulting methanol solution was dried under nitrogen and re-suspended in 50 μL of HPLC loading buffer (mobile phase A). An aliquot (20 μL) of sample solution was injected onto HPLC–MS/MS system for the quantification of seven analytes.

3. Results and discussion

3.1. Precursor and product ions selections for target analytes

The multiple-reaction-monitoring quantitative detection was used in this current method. Q1 full scan spectra of E1 and its six metabolites were first obtained by infusion of 1 μg/mL analyte standard solution into the ESI-MS/MS and APCI-MS/MS. The protonated molecular ions ($[M+H]^+$) and deprotonated molecular ions ($[M-H]^-$) were chosen as precursor ions for product ion scans in positive and negative ionization ESI-MS/MS modes, respectively. From the product ion spectrum, the most abundant ion was chose as product ion to be monitored in the MRM for quantitative detection of each analyte. In APCI-MS/MS, the protonated molecular ion ($[M+H]^+$) and deprotonated molecular ion ($[M-H]^-$) were also the most abundant ion in positive and negative ionization modes. This observation was consistent with previous reports [14,19,21]. Therefore, the same precursor and product ions were used for APCI- and ESI-MS/MS in the MRM for quantitative detection of all analytes. Table 1 lists *m/z* values of precursor and

Table 1
Monitoring ions (precursor and product ions) for multiple-reaction-monitoring mode in HPLC-MS/MS

Analyte	Precursor ion/product ion (*m/z*)	
	Positive mode	Negative mode
E1	271.1/253.0	269.1/145.0
2-OH E1	287.2/258.9	285.1/161.0
4-OH E1	287.2/258.9	285.1/161.0
16α-OH E1	287.2/250.9	285.0/158.8
2-OMe E1	301.2/283.2	299.1/284.4
4-OMe E1	301.2/283.2	299.1/284.4
2-OH-3-OMe E1	301.2/283.2	299.1/284.4
E1-d_4[a]	275.2/257.3	273.1/141.2

[a] E1-d_4: a deuterium-labeled internal standard, estrone-2,4,16,16-d_4.

product ions in positive and negative modes used for the seven analytes.

3.2. Signal response comparison of target analytes using ESI and APCI

Ionization processes of ESI and APCI are mechanistically different, so the signal responses from these two ionization processes may vary significantly. To compare the signal responses of ESI and APCI Ionization sources for each target analyte, the signal-to-noise (S/N) ratios of each target analyte in ESI-MS/MS and APCI-MS/MS were measured using flow injection (analyte concentration: 20 μL of 1 μg/mL in methanol; flow rate: 600 μL/min with the mobile phase) of analyte standard solutions into the mass spectrometer. The precursor and product ions listed in Table 1 were used as monitoring ions in MRM mode in both ESI-MS/MS and APCI-MS/MS. The signal intensities of the seven analytes were optimized using automatic tuning function provided by the instrument operating software Analyst for API 3000.

Fig. 2 shows the S/N ratios for each analyte in both positive and negative ESI- and APCI-MS/MS modes. The noise levels neighboring the analyte signals were about 500 cps. The seven analytes can be classified into three categories according to the mass spectrometric parameters that generated optimal signal quality. For group-I analytes, E1 and 16α-OH E1, both positive and negative APCI-MS/MS modes provided better S/N ratios than positive and negative ESI-MS/MS modes. The analytes of group I produced signals with the highest S/N ratios in (+)APCI-MS/MS mode and the second highest in (−)APCI-MS/MS mode. Therefore, the first general conclusion from Fig. 2 is that the (+)APCI-MS/MS mode is the most sensitive and the best choice for the analysis of group-I analytes. For group-II analytes, 2-OMe E1, 4-OMe E1, and 2-OH-3-OMe E1, (+)APCI-MS/MS provided better S/N ratios than (+)ESI-MS/MS. No signal was detected for group-II analytes in negative modes of either ESI- or APCI-MS/MS. Thus, the second general conclusion from the data in Fig. 2 is that the (+)APCI-MS/MS mode is also the most sensitive and best choice for analysis of group-II analytes. For group-III analytes, 2-OH E1 and 4-OH E1, negative ESI- and APCI-MS/MS modes provided much better S/N ratios than positive ESI- and APCI-MS/MS modes.

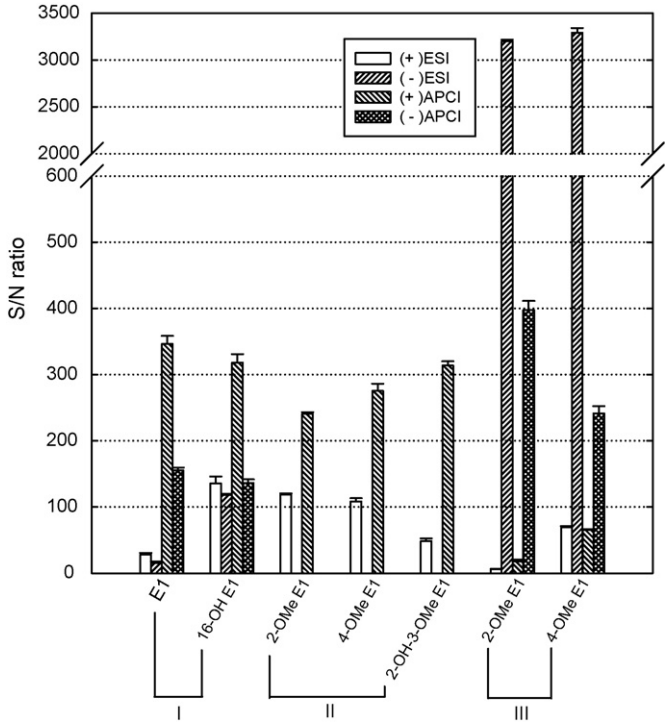

Fig. 2. Signal responses of E1 and its metabolites in four ionization modes ((+)ESI-, (−)ESI-, (+)APCI-, and (−)APCI-MS/MS). The S/N of each analyte was measured using flow injection (analyte concentration: 20 μL of 1 μg/mL in methanol; flow rate: 600 μL/min with the mobile phase) of analyte standard solutions into the mass spectrometer.

Group-III analytes produced signals with the highest S/N ratios in (−)ESI-MS/MS mode and the second highest in (−)APCI-MS/MS mode. Both of the group-III analytes have two hydroxyl groups in the A aromatic ring, therefore the analytes are more polar and more easily deprotonated than the analytes of groups I and II [22]. Therefore, the third general conclusion from Fig. 2 is that the (−)ESI-MS/MS mode is the most sensitive and best choice for analysis of group-III analytes. In summary, (+)APCI-MS/MS mode produced optimal signal quality for the analysis of group-I and group-II analytes and (−)ESI-MS/MS mode for group-III analytes.

3.3. Optimization of chromatographic conditions for 2-OH E1 and 4-OH E1

The two analytes, 2-OH E1 and 4-OH E1, could not be distinguished by mass spectrometry since the same precursor and product ions were used in MRM mode (see Table 1). Therefore, chromatographic separation was required to allow the positive identification of these two substances through the optimization of chromatographic parameters, such as chemical composition and pH of the mobile phase. Chromatographic resolution of these two analytes was used as an index to determine the efficiency of separation. A standard solution containing both the analytes, 20 μL of 1 μg/mL for each analyte, was injected onto the HPLC–ESI-MS/MS at a flow rate of 600 μL/min. Isocratic elutions with 40, 50, 55, 60, 65, 70, and 75% of methanol or

30, 35, 40, 45, 50, 55, and 60% of acetonitrile in water (v/v) were performed and the chromatographic resolution of these two analytes was determined for each mobile phase composition. For the mobile phase of methanol/water, the highest chromatographic resolution was achieved by using 60% of methanol/water. For the mobile phase of acetonitrile/water, the highest chromatographic resolution was achieved by using 40% of acetonitrile/water. The resolution achieved using the mobile phase of 40% acetonitrile/water (resolution = 1.0) was higher than that in 60% methanol/water (resolution = 0.9), and therefore the mobile phase of 40% acetonitrile/water (v/v) produced optimal chromatographic resolution for the analysis of 2-OH E1 and 4-OH E1 using HPLC-ESI-MS/MS. The dependence of pH values of the mobile phase was also investigated. Isocratic elutions with 40% of acetonitrile/water at different pH values (pH 3.5, 4.5, 5.5, 6.5, and 7) were performed and the chromatographic resolution of these two analytes was determined. The resolution decreased slightly with the pH value of the mobile phase. The highest resolution (resolution = 1.4) was obtained at pH values of 4.5 and 3.5. In summary, a mobile phase containing 40% acetonitrile/water (v/v) with a pH of 4 produced an optimal chromatographic resolution for the separation of 2-OH E1 and 4-OH E1.

3.4. Recommended analytical procedures for simultaneous detection of estrone and its six metabolites

As described in Section 3.2, the signal responses of seven target analytes in ESI- or APCI-MS/MS varied considerably. Among the four ionization modes, two analytes, 2-OH E1 and 4-OH E1, produced the best signal quality in (−)ESI-MS/MS mode, and the others in (+)APCI-MS/MS mode. However, ESI and APCI sources cannot be used in a single HPLC run using the present API 3000 mass spectrometer. The two analytes, 2-OH E1 and 4-OH E1, that produced signals with the highest S/N ratios in (−)ESI-MS/MS mode, also gave their second highest S/N ratios in (−)APCI-MS/MS mode. The S/N ratios of 2-OH E1 and 4-OH E1 produced by (−)ESI-MS/MS were 3200 ± 16 and 3290 ± 50 (mean ± standard deviation, $n = 3$) respectively, and those in (−)APCI-MS/MS were 398 ± 14 and 242 ± 11 ($n = 3$). Although the S/N ratios produced in (−)ESI-MS/MS for 2-OH E1 and 4-OH E1 were 8 (3200/398) and 14 (3290/242) times higher than those in (−)APCI-MS/MS respectively, the S/N ratios produced by (−)APCI-MS/MS for 2-OH E1 and 4-OH E1 were still higher or comparable to those for the other five analytes (E1, 16α-OH E1, 2-OMe E1, 4-OMe E1, and 2-OH-3-OMe E1) generated in either positive or negative APCI- and ESI-MS/MS. That is, although (−)ESI-MS/MS yielded best signal quality, (−)APCI-MS/MS provided sufficient signal intensity and sensitivity for the analysis of 2-OH E1 and 4-OH E1. Therefore, the APCI source was recommended for simultaneous detection of E1 and its six metabolites using HPLC-MS/MS.

The group-I (E1 and 16α-OH E1) and group-II (2-OMe E1, 4-OMe E1, and 2-OH-3-OMe E1) analytes produced better signal quality in (+)APCI-MS/MS mode and the group-III analytes (2-OH E1 and 4-OH E1) in (−)APCI-MS/MS mode (see Fig. 2).

Table 2
Analytical parameters of E1 and its metabolites

Analyte	Retention time (min)	APCI mode	Precursor/product ions (*m/z*)
16α-OH E1	5.0	Positive	287.2/250.9
2-OH E1	7.9	Negative	285.1/161.0
4-OH E1	8.9	Negative	285.1/161.0
E1-d_4[a]	13.7	Positive	275.2/257.3
E1	13.9	Positive	271.1/253.0
4-OMe E1	14.8	Positive	301.2/283.2
2-OMe E1	16.2	Positive	301.2/283.2
2-OH-3-OMe E1	18.3	Positive	301.2/283.2

[a] E1-d_4: a deuterium-labeled internal standard, estrone-2,4,16,16-d_4.

Therefore, HPLC-APCI-MS/MS with switching between positive (E1, 16α-OH E1, 2-OMe E1, 4-OMe E1, and 2-OH-3-OMe E1) and negative (2-OH E1 and 4-OH E1) modes provided optimal signal quality for simultaneous detection of E1 and its six metabolites. Programmed switching between positive and negative modes during an HPLC run is a function provided by the API 3000. The recommended parameters for simultaneous detection of E1 and its six metabolites are summarized in Table 2 and the HPLC-MS/MS selected reaction monitoring chromatogram was shown in Fig. 3.

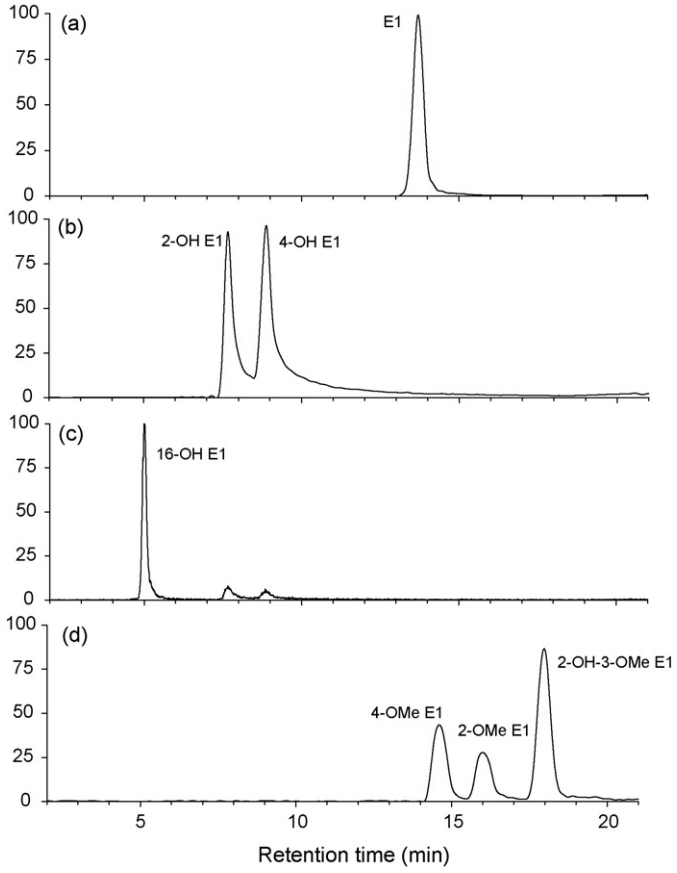

Fig. 3. A HPLC-MS/MS selected reaction monitoring chromatographic profile of estrone and its six metabolites (1 μ/mL standard solution). (a) E1, (b) 2-OH E1 and 4-OH E1, (c) 16α-OH E1, and (d) 2-OMe E1, 4-OMe E1, and 2-OH-3-OMe E1.

Table 3
Typical calibration curves obtained by the analytical method, and instrumental detection limits

Analyte	Concentration range (ng/mL)	Equations	Correlation coefficient (R^2)	Instrumental detection limit (ng/mL)
E1	10–1000	$y = 0.0121x + 0.1508$	0.998	1.4
2-OH E1	10–1000	$y = 0.0084x + 0.0248$	0.996	0.9
4-OH E1	10–1000	$y = 0.0137x - 0.0514$	0.996	0.9
16α-OH E1	10–1000	$y = 0.0026x + 0.0320$	0.997	1.4
2-OMe E1	10–1000	$y = 0.0144x + 0.1163$	0.996	1.4
4-OMe E1	10–1000	$y = 0.0082x + 0.1699$	0.998	1.4
2-OH-3-OMe E1	10–1000	$y = 0.0162x + 0.1792$	0.997	1.4

3.5. Calibration curves, detection limits, precision, and matrix effect

Calibration curves for the seven analytes were established using standard solutions at concentrations of 0, 10, 50, 100, 200, 500, 750, and 1000 ng/mL and a deuterium-labeled internal standard (E1-d_4). Not all of the deuterium-labeled internal standards for seven analytes were available. Therefore one deuterium-labeled standard was chosen as the internal standard for all analytes in this method. The calibration curves for the analytes were constructed from the peak area (counts × time) in MRM mode, to give a regression equation $y = ax + b$, where y is the signal intensity ratio between analyte and deuterium-labeled internal standard (E1-d_4), x is the concentration of analyte standard solution in ng/mL, a is the slope, and b is the intercept. Table 3 summarizes typical calibration curves for the seven analytes obtained by the aforementioned analytical procedures. The response was linear over the concentration range (10–1000 ng/mL) for all the analytes. The correlation coefficients for the seven analytes ranged from 0.995 to 1.000.

To determine the detection limits, standard solutions containing analytes were prepared at varying concentrations by serial dilution (by a factor of 2). Each neat standard solution of analyte was injected onto the HPLC-APCI-MS/MS system to determine the lowest concentration of the analyte standard solution that could give a detectable signal. Using a criterion of S/N ratio greater than 3, the instrumental detection limits for pure standard solutions containing target analytes ranged from 0.9 to 1.4 ng/mL (Table 3). The detection limits reported in this manuscript are not better than those in some recently published articles [16,17] and insufficient for measurement of the steroids in most body fluids. Nevertheless, the procedures reported here could be utilized for in-vitro experiments as well as for evaluation of samples collected from special physiological conditions such as pregnancy. The usefulness of the proposed analytical scheme for simultaneous detection of E1 and its six metabolites was demonstrated with applications of the method to cell culture medium (Section 3.6).

Standard solutions of seven analytes in 50, 200, 1000 ng/mL were used to evaluate the intra-day instrumental precision by six-repeated injections of the same concentration using the HPLC–APCI-MS/MS system described above. Relative standard deviations (RSD) for the six replicate analyses of the seven analytes at three concentrations were used to evaluate the instrumental precision, resulting RSD values ranged from 4.5 to 7.5% (Table 4).

Table 4
Instrumental precision of HPLC–APCI-MS/MS measurements of E1 and its six metabolites

Analyste	Instrumental precision relative standard deviations ($n = 6$)			
	50 (ng/mL)	200 (ng/mL)	1000 (ng/mL)	Mean
E1	14%	5.0%	3.8%	7.5%
2-OH E1	4.7%	7.0%	1.7%	4.5%
4-OH E1	4.8%	9.0%	2.8%	5.5%
16α-OH E1	7.5%	3.9%	9.3%	6.9%
2-OMe E1	3.1%	7.2%	4.1%	4.8%
4-OMe E1	6.5%	9.4%	2.1%	7.0%
2-OH-3-OMe E1	6.5%	6.4%	3.5%	5.4%

The matrix effect and the possibility of ionization suppression or enhancement was evaluated by comparing the intensity of the analyte standards and standards spiked into cell culture medium extracts [23]. The response of the analyte standards spiked in to cell culture medium after extraction divided by the response of the same analyte standards was used as an index (matrix effect factor, MEF) to quantify the degree of matrix effects. MEF (%) = $B/A \times 100$, where A is the response of a neat standard solution, and B is the response of the same standard solution spiked post-extraction. A value of >100% indicates ionization enhancement, and a value of <100% indicates ionization suppression. Table 5 shows the MEFs for analysis of E1 and its metabolites in cell culture medium using the recommended analytical procedures (Section 3.4). There is little matrix effect

Table 5
Matrix effect factors for cell culture medium

Analyste	Matrix effect factor[a] ($n = 3$)	
	Mean (%)	Standard deviation (%)
E1	112	12
2-OH E1	36	18
4-OH E1	75	17
16α-OH E1	110	13
2-OMe E1	104	21
4-OMe E1	105	18
2-OH-3-OMe E1	91	15

[a] Matrix effect factor expressed as the ratio of the mean peak area of an analyte spiked post-extraction to the mean peak area of the same analyte standards multiplied by 100.

for all analytes except for 2-OH E1 (MEF = 36%) and 4-OH E1 (MEF = 75%). The low MEFs of 2-OH E1 and 4-OH E1 may be caused by ionization suppression of co-elute matrix.

3.6. Quantitative detection of E1 and its metabolites in cell culture medium

The usefulness of the proposed analytical scheme for simultaneous detection of E1 and its six metabolites was demonstrated with applications of the method to cell culture medium. Human Hep G2 cells were treated with 2,3,7,8-TCDD as described in Section 2.3. Aliquots of cell culture medium were collected and analyzed by HPLC–APCI-MS/MS. Fig. 4 shows the levels of the analytes in the cell culture medium treated with E1 and varying amounts of 2,3,7,8-TCDD for 24 h. The concentrations of E1 and its metabolites in the cell culture medium vary with the amounts of 2,3,7,8-TCDD added. E1 levels decreased when the cell culture medium was treated with 2,3,7,8-TCDD increased (Fig. 4(a)). It is likely that E1 was converted into its metabolites, 2-OH E1, 4-OH E1, 16α-OH E1, 2-OMe E1, 4-OMe E1, and 2-OH-3-OMe E1, by cytochrome P450 and catechol *O*-methyltransferase (COMT) enzymes, as shown in Fig. 1.

E1 was possibly converted into its hydroxyl metabolites, 2-OH E1, 4-OH E1, 16α-OH E1, and the relative amount of E1 and its metabolites in the cell culture medium will vary with the amounts of 2,3,7,8-TCDD added. Fig. 4(b) shows that the levels of 2-OH E1 and 4-OH E1 in 2,3,7,8-TCDD-treated cell culture medium were higher than those in untreated cell culture medium and increased with the amount of 2,3,7,8-TCDD increased. The levels of 2-OH E1 and 4-OH E1 in cell culture medium treated with 10 nM 2,3,7,8-TCDD were 4.6 (22/4.7) and 1.9 (5.0/2.7) times higher than those in untreated medium, respectively. The levels of 16α-OH E1 in 2,3,7,8-TCDD-treated cell culture medium were lower than that in untreated cell culture medium and decreased slightly with the amount of 2,3,7,8-TCDD increased (Fig. 4(a)). The toxicant 2,3,7,8-TCDD is the most potent known cytochrome P450 1A1 inducer [24]. These observations, i.e. levels of 2-OH E1 and 4-OH E1 increased with the amount of 2,3,7,8-TCDD increased while 16α-OH E1 decreased, are consistent with previous reports [24,25]. The cytochrome P450 1A1 exhibits relatively different catalytic activities for E1 hydroxylation at C2, C4, and C16 [25]. It has the highest activity for E1 hydroxylation at C2, and the second highest for E1 hydroxylation at C4. For E1 hydroxylation at C16, the cytochrome P450 1A1 has a low activity and these activities correlate with the present observations.

As shown in Fig. 1, 2-OH E1 and 4-OH E1 were converted into metabolites, 2-OMe E1 and 4-OMe E1, by catechol *O*-methyltransferase (COMT) respectively, and the levels of the metabolites in the cell culture medium also vary with the amounts of 2,3,7,8-TCDD added. Fig. 4(c) shows that the levels of 2-OMe E1 and 4-OMe E1 in 2,3,7,8-TCDD-treated cell culture medium were higher than that in untreated cell culture medium and increased with the amount of 2,3,7,8-TCDD increased. The levels of 2-OMe E1 and 4-OMe E1 in cell culture medium treated with 10 nM 2,3,7,8-TCDD were 7.7 (130/17) and 36 (16/0.45) times higher than those of untreated medium, respectively. There are at least two possible explanations for these observations. One possibility to be considered is that the increased levels of 2-OH E1 and 4-OH E1 may lead to a rapid subsequent metabolism, 2-OH-E2 to 2-MeO-E2, and 4-

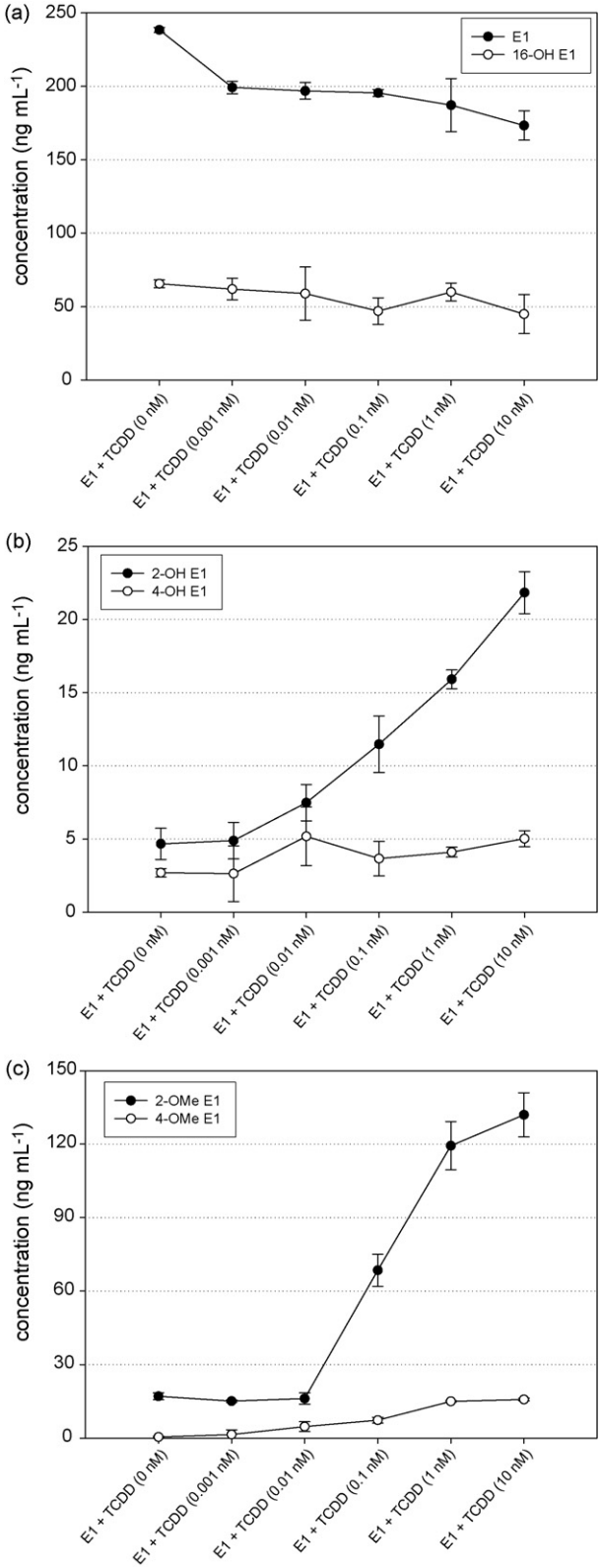

Fig. 4. The levels of E1 and its metabolites in Human Hep G2 cells added by different amounts of 2,3,7,8-TCDD.

OH-E2 to 4-MeO-E2. Another possibility to be considered is that 2,3,7,8-TCDD treated largely increased the capability of COMT to metabolize 2-OH-E2 to 2-MeO-E2, and 4-OH-E2 to 4-MeO-E2. The association between the activity of the COMT enzyme and the amount of 2,3,7,8-TCDD added require further investigation. The level of 2-OH-3-OMe E1 was below the detection limit in all samples. All target analytes were below their detection limits in blank cell culture medium (untreated with E1 and/or 2,3,7,8-TCDD) or DMSO solution indicating no background interference for the target analytes.

In our opinions, this report, provides some new information to readers as well as those who are interested in the interactions between 2,3,7,8-TCDD and estrogen metabolism. In the previously published reports, most studies investigated on altered E2 metabolism caused by 2,3,7,8-TCDD [26–29]. Three publications reported the association between 2,3,7,8-TCDD and E1 metabolism, however, those studies only measured one or up to three metabolites, not all six metabolites reported in this manuscript [29–31]. Our data presented a whole picture portraying 2,3,7,8-TCDD caused an extensive alteration of E1 metabolism in human Hep G2 cells. The levels of four E1 metabolites (2-OH E1, 4-OH E1, 2-OMe E1, and 4-OMeE1) in cell culture medium increased with the amount of 2,3,7,8-TCDD increased (Fig. 4(b) and (c)) and the level of 16α-OH E1 decreased with the amount of 2,3,7,8-TCDD increased (Fig. 4(a)).

4. Conclusion

We report here a systematic comparison of different ionization modes ((+)ESI, (−)ESI, (+)APCI and (−)APCI) for the detection of E1 and its metabolites. Among the four ionization modes (+)APCI-MS/MS mode produced optimal signal quality for E1,16α-OH E1, 2-OMe E1, 4-OMe E1, and 2-OH-3-OMe E1 and (−)ESI-MS/MS for 2-OH E1 and 4-OH E1. Furthermore, HPLC-APCI-MS/MS with switching between positive (E1, 16α-OH E1, 2-OMe E1, 4-OMe E1, and 2-OH-3-OMe E1) and negative (2-OH E1 and 4-OH E1) modes is recommended for simultaneous detection of E1 and its six metabolites. The proposed analytical scheme could provide useful information in investigations for quantitative profiling of estrogens and their metabolites that may be related to carcinogenesis, reproduction, sexual development, and effects of endocrine disruptor exposure on the estrogen metabolism.

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