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Quantitative analysis of estrogens and estrogen metabolites in endogenous MCF-7 breast cancer cells by liquid chromatography–tandem mass spectrometry

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ABSTRACT

To study the roles of estrogens and estrogen metabolites (EMs) in breast carcinogenesis, we reported a quantitative liquid chromatography–tandem mass spectrometry (LC–MS/MS) method utilizing selective reaction mode (SRM) to analyze estrogens and EMs in the extracellular and intracellular compartments of endogenous MCF-7 breast cancer cells through simple ethyl acetate (EA) extraction and dansyl chloride derivatization. Under a 35-min LC gradient elution on a reversed phase C18 column, the method was shown to simultaneously quantify 12 estrogens and EMs: estrone (E1) and its 2-, 4-, 16α-hydroxy derivatives (2-OHE1, 4-OHE1, 16α-OHE1), and 2-, 4-methoxy derivatives (2-MeOE1, 4-MeOE1); 17β-estradiol (E2) and its 2-, 4-hydroxy derivative (2-OHE2, 4-OHE2) and 2- and 4-methoxy derivatives (2-MeOE2 and 4-MeOE2); and estriol (E3), using ethinylestradiol (EE2) as the internal standard (IS). Using a calibration curve–standard addition hybrid method, we were able to determine the amount of estrogens and EMs in not only the treated cells but also the non-treated cells. The limits of quantification (LOQs) were determined to range from 0.05–80 pg on column with an inter-batch accuracy around 72–123% and precision around 1–10%. Results indicated that trace amounts (<0.9 fg/cell) of E1 and E2 were present in both the extra- and intra-cellular compartments under non-treated condition but DMSO could induce E1 and E2 as well as trace amounts (<2.25 fg/cell) of EMs in the cell. E2 treatment substantially increased not only E1 and E2 in the intra-cellular (60 fg/cell) and extra-cellular (3000 fg/cell) compartment but also substantially induced EMs primarily in the extracellular compartment (0.6–25 fg/cell). These data implied that EMs could be quickly generated and distributed to the extracellular compartment by E2 within 24 h of treatment and DMSO solvent could potentially induce slight estrogen effects.

1. Introduction

Estrogens and estrogen metabolites (EMs) produced by endogenous conversion of estrogens are known to play important roles in the development and progression of breast cancer [1–4]. Hydroxylation at the C-2 and C-4 position of 17β-estradiol (E2) yields the catecholestrogens (CEs), 2-hydroxyestrone (2-OHE1) and 2-hydroxyestradiol (2-OHE2), 4-hydroxyestrone (4-OHE1) and 4-hydroxyestradiol (4-OHE2) while hydroxylation at the C-16 α position yields 16α-hydroxyestrone (16α-OHE1) which is subsequently converted to estriol (E3) [5,6]. The hydroxylated products exert very different biological properties: the 16 α-hydroxy and 4-hydroxy metabolites are active estrogens whereas the 2-hydroxy metabolites are not as active [7,8]. However, the binding and redox cycling activities of CEs can be blocked *via* *O*-methylation by catechol-O-methyltransferase (COMT) which converts 2-OHE1/E2 and 4-OHE1/E2 to their methoxy derivatives 2-MeOHE1, 2-MeOHE2, 4-MeOHE1, and 4-MeOHE2, respectively [9–13]. Some of the EMs are released to the extracellular compartment and are subsequently excluded to the circulating fluids such as serum or urine if they are in a living body. Thus, analytical methods to reliably quantify individual estrogens and EMs in extracellular and intracellular compartment are essential tools in studying the genotoxic effect of estrogens.

Gas chromatography–mass spectrometry (GC/MS) method using negative chemical ionization with pentafluoropropionic anhydride derivatization was reported for quantitatively measuring endogenous estrogens and EMs in late pregnancy human plasma and rat plasma spiked with these compounds [14]. Liquid chromatography–atmospheric pressure chemical ionization-tandem mass spectrometry (LC–APCI-MSMS) method has also been reported for measuring 2-methoxyestradiol in human plasma from a cancer patient who received a single oral dose (2200 mg) of 2-methoxyestradiol [15]. Furthermore, high performance liquid chromatography–electrospray ionization-tandem mass spectrometry (HPLC–ESI-MSMS) method that uses simple hydrolysis and derivatization is reported for measuring endogenous estrogens and EMs in urine [16] and in serum [17] from premenopausal and

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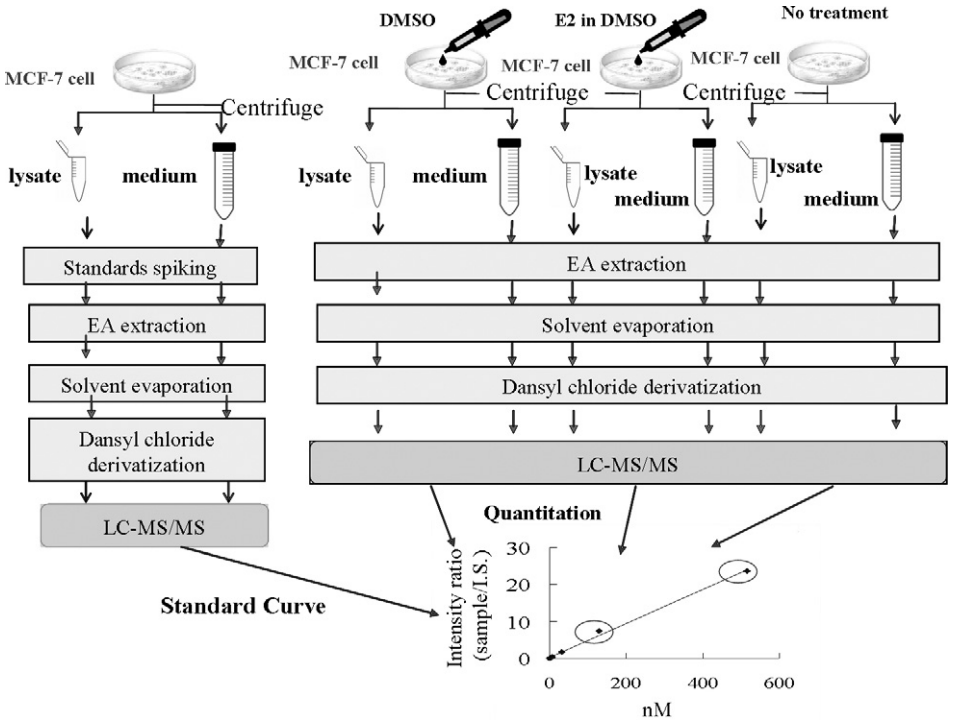

Fig. 1. Workflows for sample preparation.

postmenopausal women. These samples were prepared in sequential steps which include organic solvent extraction or solid phase extraction [18].

For the detection of estrogens and EMs in a culture cell, radio- or enzyme immunoassay is still the most popular technique [19–22]. However, the immunoassay is likely to suffer from poor specificity, accuracy, and reproducibility due to cross-reactivity and lot-to-lot variation of antibodies. A few MS-based methods have been developed to detect EMs in a culture cell. ESI and APCI-MSMS method operated under both positive and negative ionization modes was used to detect E1, 16α-hydroxyestrone, 2-methoxyestrone, 4-methoxyestrone, and 2-hydroxyestrone-3-methyl in the cell culture medium of human liver carcinoma cells [23]. HPLC–ESI/MSMS method coupled with a post column infusion of the internal standard (IS) was developed to investigate steroids in yeast-mediated cell culture medium [24]. Whereas, these methods were only for EMs present in the cell medium but not for EMs present in the intracellular compartment which is expected to have much less amount of EMs. In this report, we aim to develop a LC–ESI-MSMS method to measure estrogens and EMs in the extracellular (cell culture medium) as well as in the intracellular (cell lysate) compartment. Dansyl chloride derivatization [17,25] will be adopted to couple with MS detection under selective reaction mode (SRM). Moreover, the developed method will be validated following the reported guidelines [26] and then applied for investigating the endogenous estrogens and EMs in MCF-7 cells under no treatment as well as under the treatment of the organic solvent and 17β-estradiol.

2. Experimental

2.1. Chemicals and reagents

Estrone (≥99%), 17β-estradiol (≥99%), estriol (≥99%), 4-hydroxyestrone (≥98%), 2-hydroxyestradiol (≥95,5%), 4-hydroxyestradiol (≥95.5%), 2-methoxyestradiol (≥98%), 16α-hydroxyestrone (≥99%), ethinylestradiol (≥98%), dansyl chloride (≥99%), sodium orthovanadate (≥90%), leupeptin hemisulfate salt sodium orthovanadate (≥94%), dimethyl sulfoxide (DMSO) (≥99.5%), and Dulbecco's modified eagle medium (DMEM) were purchased from Sigma–Aldrich (St. Louis, USA). 2-Hydroxyestrone (≥98%), 2-methoxyestrone (≥98%), 4-methoxyestrone (≥90%) and 4-methoxyestradiol (≥99%) were purchased from STERALOIDS (Newport, RI, USA). Acetone (≥99.5%), methanol (≥99.9%) and ethyl acetate (EA) (≥99.5%) were purchased from Mallinckrodt Baker (Phillipsburg, NJ). Acetonitrile (ACN) (≥99.5%), sodium bicarbonate (99–100%), L-ascorbic acid (≥99.8%), and sodium hydroxide (≥98.8%) were from J.T. Baker (Phillipsburg, NJ). Formic acid (FA) (≥96.0%) and sodium acetate (≥99%) were from Riedel-de Haën (Steinheim, Germany). Trypsin (200 U/mL), streptomycin (200 U/mL) and fetal bovine serum (FBS) were from Invitrogen (GibcoBRL, Gaithersburg, USA). Phenylmethanesulfonyl fluoride (PMSF) (≥98.8%) was purchased from Fluka (Buchs, Switzerland) and dithiothreitol (DTT) (≥99.5%) was from J.T. Baker (Quebec, Canada). β-Glucuronidase/sulfatase (type H-2, ≥2000 units sulfatase activity and ≥100,000 units β-glucuronidase activity) was from Helix Pomatia (St. Louis, USA) and penicillin was purchased from GibcoBRL (Gaithersburg, USA).

2.2. Cell culture

MCF-7 cells were cultured in DMEM medium in 144 mm × 21 mm dishes (15 mL or 10^6 cells per dish) supplemented with 10% FBS, 1% penicillin, and 5% CO_2 at 37 °C. For E2 treatment, the cells were cultured for an additional 24 h in the absence of serum, followed by the treatment with 10 μL of 1 μM E2 dissolved in DMSO for 24 h at 37 °C. The cells were also treated with the same volume (10 μL) of DMSO as the control. A total of 15 mL of the cell medium was collected and L-ascorbic acid was added to the medium at a final concentration of 0.1% before the storage at −20 °C. For collecting the cell lysate, the treated cells were first washed three times in ice cold phosphate-buffered saline (PBS), trypsinized with 5% trypsin–EDTA in PBS at 1:1 volume ratio, incubated at 37 °C for 5 min, and then added with an equal volume of the culture medium to stop the reaction. The solution was

Table 1
Reproducibility of the LC retention time.

Analytes	Ion pair	Retention time (min)	RSD (%), $n = 3$
E3	522/156	13.07	0.27
16α-OHE1	520/171	14.62	0.35
2-MeOE2	536/171	18.27	0.34
4-MeOE2	536/171	18.88	0.32
E2	506/171	19.33	0.32
2-MeOE1	534/171	20.13	0.31
4-MeOE1	534/171	21.07	0.34
EE2	530/171	20.75	0.33
E1	504/171	21.33	0.30
4-OHE2	755/521	26.14	0.21
2-OHE2	755/521	27.46	0.29
4-OHE1	753/170	29.20	0.21
2-OHE1	753/170	29.85	0.25

then centrifuged under 600 × *g* for 5 min at 4 °C. After removing the supernatant, the cells were washed with the ice-cold PBS twice and then dissolved in 1 mL PBS composed of 0.5 mM PMSF, 2 μg/mL leupeptin, 0.5 mM DTT, 1 mM sodium orthovanadate, and 0.1% L-ascorbic acid. The re-dissolved cell solution was sonicated (digital sonifier: Branson, NY, USA) under 40% power for 5 s and then stopped for 3 s; the cycle was repeated up to a total time of 90 s. The lysed cells were centrifuged under 13,800 × *g* for 10 min at 4 °C. The resulting solution was the cell lysate and was stored at −80 °C until use.

2.3. Sample preparations, extraction and derivatization

The stock solution (around 1 mM) of estrogens and EMs as well as the EE2 (IS) were prepared in methanol for constructing the calibration curves (the left of Fig. 1). The stock mixture solution (1 mL) was prepared by mixing 12 estrogens/EMs and L-ascorbic acid in methanol at a final concentration ranging from 750 to 0.48 μM for each estrogens and EMs and 0.1% for L-ascorbic acid. Working standard mixtures were prepared by a series of dilutions from the stock mixture. For optimizing the instrument conditions, a test solution was prepared by mixing 100 μL of each working standard mixture with 100 μL of sodium bicarbonate buffer (0.1 M, pH9.0) and 100 μL of dansyl chloride (1 mg/mL in acetone) and then the solution was incubated at 60 °C for 15 min. On the other hand, 200 μL of each working standard mixture and 10 μL of 278 μM IS was spiked into the cell medium (14,800 μL without E2 treatment), which yielded a final concentration of 10,000–6.4 nM for each standard and 0.19 nM for the IS in the cell medium. Meanwhile, 200 μL of each working standard mixture and 10 μL of 278 μM IS was spiked into the cell lysate (800 μL without E2 treatment), which yielded a final concentration of 1000–0.047 nM for each standard and 2.8 nM for the IS in the cell lysate. An equal volume of methanol was also spiked into the cell medium or cell lysate for background subtraction. The spiked cell medium or cell lysate standard mixtures (including the methanol spiked background solutions) were immediately extracted with EA and derivatized with dansyl chloride to minimize the occurrence of conjugation reaction. The spiked cell medium standard mixtures (15 mL) were extracted with 22.5 mL of EA for 3 times and each spiked cell lysate standard mixtures (1.0 mL) were extracted with 1.5 mL EA for 3 times. After the extraction, EA was evaporated by a stream of nitrogen gas and the dried samples were re-dissolved in 100 μL 0.1 M sodium bicarbonate buffer (pH 9.0) and 100 μL dansyl chloride (1 mg/mL in acetone); the solution was then incubated at 60 °C for 15 min [21,23]. After the reaction, the samples were dried by a stream of nitrogen gas and stored at −20 °C. Before use, the dried samples were re-dissolved in 200 μL of deionized (DI) water containing 10% methanol. The recovery yield of EA extraction was determined by comparing the peak area obtained from the injection of the spiked working standard mixtures after

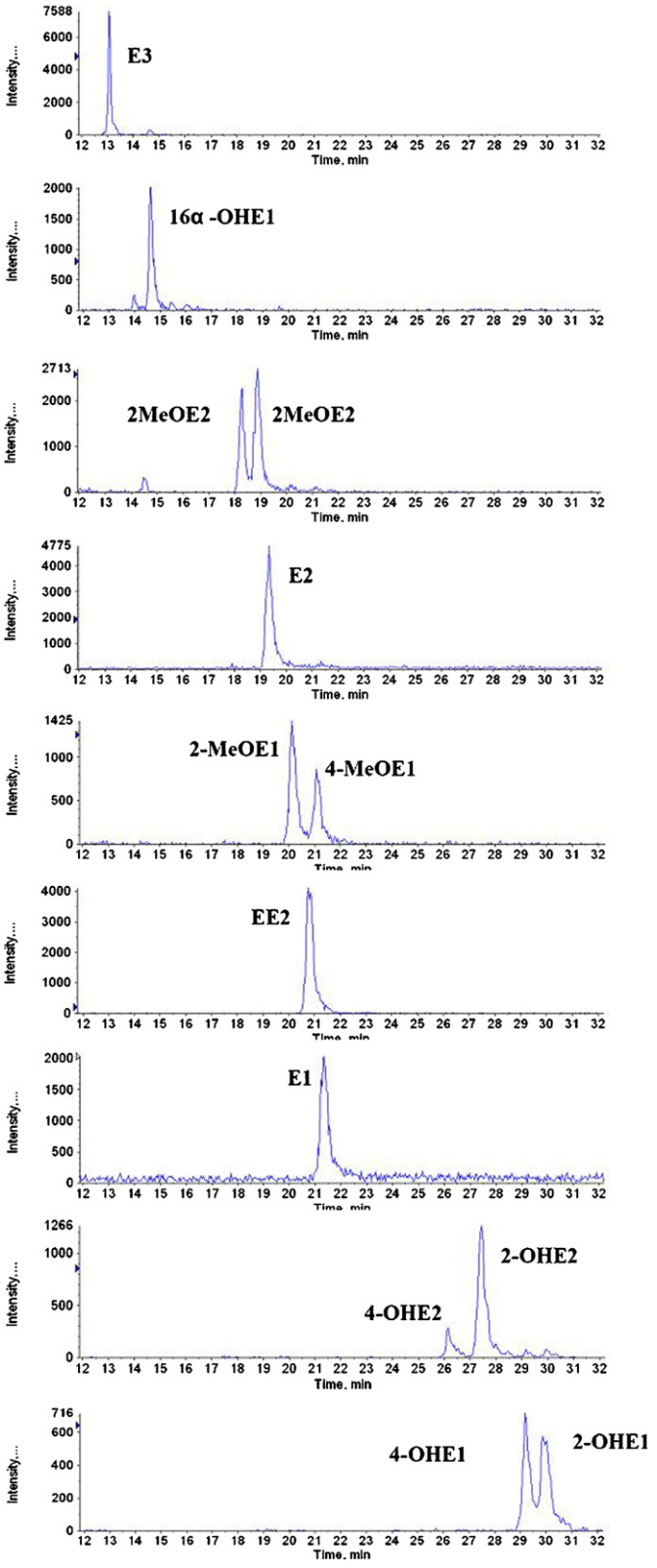

Fig. 2. SRM chromatograms of the estrogens and EMs standards. The concentration of each standard was 1.3×10^{-8} M in methanol. Precursor/product ion pairs were 522/156 for E3, 520/171 for 16α-OHE1, 536/171 for 2-MeOE2 and 4-MeOE2, 506/171 for E2, 534/171 for 2-MeOE1 and 4-MeOE1, 530/171 for EE2, 504/171 for E1, 755/521 for 4-OHE2 and 2-OHE2, 753/170 for 4-OHE1 and 2-OHE1.

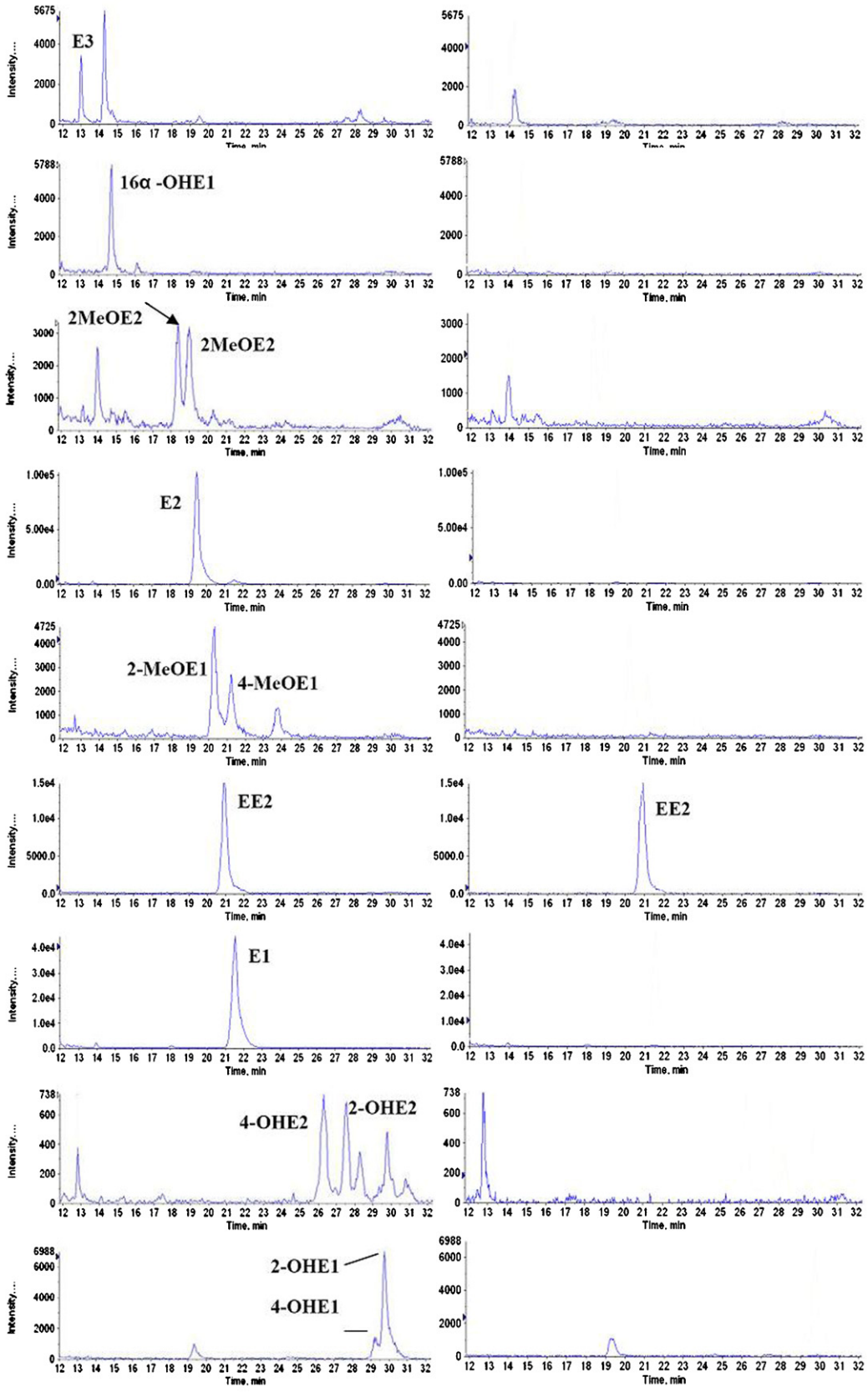

Fig. 3. The left panels are SRM chromatograms of the estrogens and EMs standards spiked in the cell medium. The final spiked concentration was 11 nM for E1 and E2, 0.3 nM for the rest of EMs. Precursor/product ion pairs were the same as shown in this figure. The right panels are the background SRM chromatograms of the non-treated cell medium which was only spiked with methanol (about 1.3%, v/v) and the IS EE2 (0.19 nM).

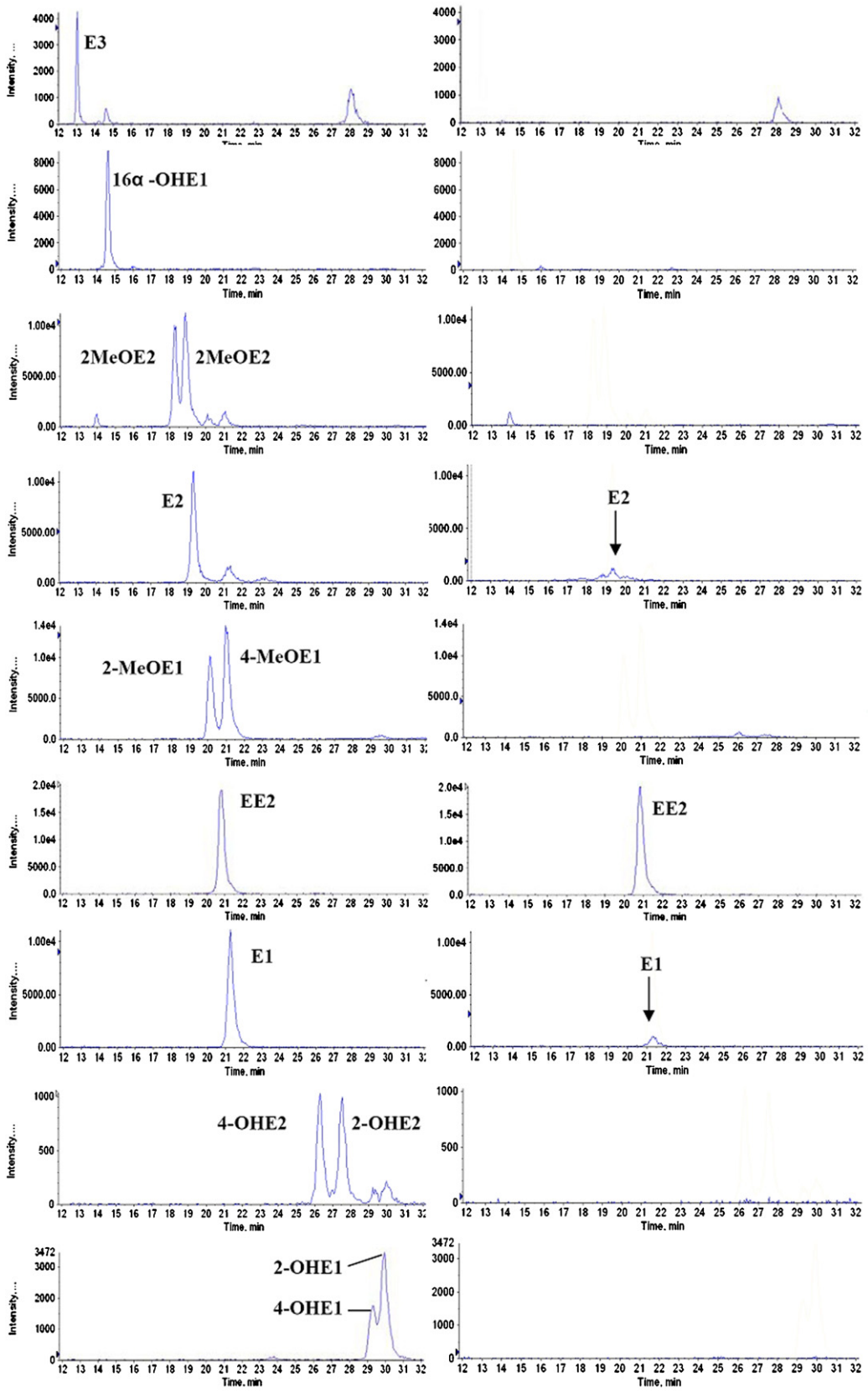

Fig. 4. The left panels are SRM chromatograms of the estrogens and EMs standards spiked in the cell lysate. The final spiked concentration was 2 nM for each standard. Precursor/product ion pairs were the same as shown in Fig. 3. The right panels are the background SRM chromatogram of the non-treated cell lysate which was only spiked with methanol (about 25%, v/v) and the IS EE2 (2.3 nM). Note that E1 and E2 were detected from the non-spiked background lysate (right).

extraction and from the working standard mixtures prepared in methanol without extraction.

2.4. Method validation

For method validation, the QC mixtures containing 0.07–1.62 nM of each estrogen/EMs and 0.19 nM IS spiked in the cell medium and 0.47–2.07 nM of each estrogen/EMs and 2.3 nM IS spiked in the cell lysate were prepared. The accuracy and precision of the method were calculated from the measurements of the QC mixtures. For the measurement, three aliquots of the same sample were analyzed, with each taken through complete sample processing and data acquisition. Moreover, to confirm whether or not the spiked samples were converted to the conjugated forms, separate sets of spiked standard mixtures were hydrolyzed by β-glucuronidase/sulfatase [20,21] followed by EA extraction and dansyl chloride derivatization; samples without hydrolyzation were also analyzed simultaneously for comparison. Once the HPLC and MS conditions for resolving and quantifying the 12 EMs and IS in spiked cell medium and spiked cell lysate were established and validated (the left of Fig. 1), we followed the workflow depicted in the right of Fig. 1 to measure the levels of estrogens and EMs in the cell medium and cell lysate under the non-treated condition as well as under a 24-h treatment of DMSO or 1 μM E2 dissolved in DMSO. Each measurement was repeated for at least 3 times.

2.5. HPLC–ESI-MS/MS

HPLC–ESI-MS/MS analysis was performed using a mass spectrometer (4000 QTRAP, Applied Biosystem, MDS Sciex, Toronto, Canada) equipped with a Turbo V ion spray source coupled with a HPLC system (Agilent 1200, CA, USA) using spiked EE2 as the IS and using SRM for detection. The precursor/product ion pairs which exhibit the highest sensitivity and can uniquely distinguish each compound from others were chosen for SRM detection. Normally, Both the HPLC and mass spectrometer were controlled by Analyst 1.4.2 software from Applied Biosystems (Darmstadt, Germany). HPLC was carried out on a C18 column (Thermo scientific; 30 mm × 2.1 mm, particle size 1.9 μm) at a flow-rate of 0.25 mL/min. Two buffers were used for the gradient elution: solvent A, 0.1% FA in DI water, and solvent B, 0.1% FA in ACN. The extracted samples were injected into a 30-μL sample loop and the elution was performed under a 35-min gradient (Supplement Table S1). Mass spectrometer conditions were set up as follows: positive ionization mode with 4500 V ionization source voltage and 350 °C source temperature. Nitrogen was used as the drying, nebulizing, and collision gas with the following setting: curtain gas 10, collision gas 12, ion Source gas 1:40, and ion Source gas 2:60.

3. Results and discussion

3.1. LC–MS conditions

To establish the analytical parameters required for LC–MS/MS measurement, 30 μL of the dansyl chloride derivatized standard mixture composed of 12 un-conjugated EMs and IS (EES) was injected into the column and analyzed by HPLC–ESI-MS/MS. Since SRM mode could help to differentiate co-eluted compounds by mass, we tried to optimize the gradient slope in order to resolve the isobaric peaks under their elution window as well as to accelerate the whole elution speed. We had attempted both methanol and ACN as the eluting organic solvent but methanol could not resolve isobaric pairs such as 4-OHE2 and 2-OHE2 that could not be resolved by MS either. In contrast, ACN could easily resolve isobaric pairs by adjusting the gradient slope that fell within the elution

Table 2
Figures of merit for the measurements of estrogens and EMs in MCF7 cell medium and cell lysate.

	Cell medium				Cell lysate			
	LOD (nM) ($S - S_{back}$)/N = 3 or S/N = 3	Range of calibration curves #LOQ	0.07–1.62 nM		LOD (nM) ($S - S_{back}$)/N = 3 or S/N = 3*	Range of calibration curves #LOQ	0.47–2.07 nM	
			Accuracy	Precision (n = 3)			Accuracy	Precision (n = 3)
E1	0.010*	9.77 nM# to 2.50 μM	83%	7%	0.018*	53.6 pM# to 54.8 nM	77%	4%
E2	0.064*	9.77 nM# to 2.50 μM	99%	5%	0.022*	53.6pM# to 54.8 nM	99%	1%
E3	0.0041	6.24 pM* to 6.39 nM	102%	10%	0.020	46.8 pM# to 47.9 nM	95%	7%
16α-OHE1	0.0082	21.6 pM# to 22.1 nM	98%	8%	0.041	81.0 pM# to 82.9 nM	105%	7%
2-MeOE1	0.017	39.6 pM# to 40.6 nM	100%	4%	0.090	100 pM# to 100 nM	110%	3%
4-MeOE1	0.012	39.6 pM# to 40.6 nM	91%	9%	0.050	100 pM# to 100 nM	92%	1%
2-MeOE2	0.020	88.0 pM# to 90.1 nM	109%	7%	0.030	200 pM# to 200 nM	123%	5%
4-MeOE2	0.010	88.0 pM# to 90.1 nM	109%	6%	0.040	200 pM# to 200 nM	112%	2%
2-OHE1	0.022	67.2 pM# to 68.8 nM	84%	9%	0.070	500 pM# to 500 nM	112%	10%
4-OHE1	0.040	67.2 pM# to 68.8 nM	116%	8%	0.130	1.00 nM# to 1.00 μM	93%	4%
2-OHE2	0.018	67.2 pM# to 68.8 nM	72%	10%	0.150	500 pM# to 500 nM	100%	4%
4-OHE2	0.034	67.2 pM# to 68.8 nM	94%	6%	0.300	500 pM# to 500 nM	125%	5%

*LOD defined at S/N = 3; #LOQ defined as the lowest concentration of the calibration curve.

Table 3
Concentrations of estrogens and EMs in MCF cell medium and lysate under non-treated and DMSO/E2-treated conditions.

Cellular condition	Medium (nM[a]), $n=3$			Lysate (nM[a]), $n=3$		
	E2 (1 μM)	DMSO (10 μL)	No treatment	E2 (1 μM)	DMSO (10 μL)	No treatment
E1	178.74 ± 7%	0.04 ± 7%	0.05 ± 5%	1.75 ± 2%	7.31 ± 4%	0.08 ± 9%
E2	673.92 ± 8%	0.10 ± 33%	0.15 ± 7%	12.90 ± 4%	1.78 ± 7%	0.17 ± 15%
E3	1.50 ± 4%	ND	ND	ND	ND	ND
16α-OHE1	ND	ND	ND	ND	ND	ND
2-MeOE1	0.14 ± 1%	ND	ND	ND	ND	ND
4-MeOE1	0.69 ± 6%	ND	ND	ND	ND	ND
2-MeOE2	1.13 ± 4%	0.02 ± 11%	ND	0.04 ± 5%	0.03 ± 10%	ND
4-MeOE2	5.66 ± 6%	0.01 ± 12%	ND	0.05 ± 6%	0.04 ± 8%	ND
2-OHE1	0.76 ± 6%	ND	ND	ND	ND	ND
4-OHE1	0.59 ± 7%	ND	ND	ND	ND	ND
2-OHE2	1.34 ± 9%	0.15 ± 18%	ND	0.29 ± 18%	0.25 ± 15%	ND
4-OHE2	1.79 ± 6%	0.25 ± 11%	ND	0.48 ± 10%	0.48 ± 7%	N/D

ND: non-detected.
[a] nM can be converted to fg/cell by multiplying a factor of 4.5 (see Section 3).

window of the isobaric pair. With the use of an optimized ACN/0.1% FA gradient (Supplement Table S1), the chromatographic profile (Fig. 2) resulting from this analysis indicated that all 13 EMs and IS were resolved ($R > 1.0$) by reversed phase C18 chromatography within 35 min using optimized MS parameters (Supplement Table S2). Notably, the isobaric pairs, 4-MeOE2/2-MeOE2, 4-MeOE1/2-MeOE1, 4-OHE2/2-OHE2, and 4-OHE1/2-OHE1 could all be resolved (Fig. 2) under the optimized elution condition. Moreover, the retention time of each analyte was reproducible under the optimized elution program with RSD < 0.40% (Table 1). Although the retention times for some pair of analytes such as 4-MeOE2 and E2 differ by less than 1 min, the use of SRM detection allows them to be differentiated and thus, has greatly reduced the task required to further optimize the chromatographic elution method.

Dansyl chloride derivative exhibited a reporter fragment ion m/z 171 which can be found in the MSMS spectra of all compounds. However, in addition to m/z 171 fragment, we have included at least one more ion pair for each compound to increase the detection specificity (Supplement Table S2). The precursor/product ion pairs used for quantification for each compounds were shown in Table 1 and Supplement Table S2. To further investigate the matrix effect and to establish analytical parameters for quantitative measurement, the spiked cell medium/lysate standard mixtures were injected into the column and analyzed by HPLC–ESI-MS/MS operating under the optimized SRM mode. We compared the signal level of the standards prepared in methanol and the standards spiked in the cell medium or cell lysate under the same concentrations. In almost every case, the peak shapes showed excellent symmetry and the difference in the signal level detected from EM-dansyl eluted regions for standards prepared in methanol and in spiked matrix was in-distinguishable when considering 20% of the recovery rate for extraction (see below). Furthermore, the chromatograms acquired under SRM conditions for each spiked estrogen and EMs standards depicted in Fig. 3 (cell medium) and Fig. 4 (cell lysate) were shown to exhibit almost background resolution. Although the resolution of 4-OHE2 and 2-OHE2 for the spiked standards was slightly decreased ($R = 0.8$ under SRM), the method is still capable of simultaneously quantifying 12 estrogens and EMs in MCF-7 cells. Moreover, no background (or matrix) signal acquired from the non-treated cell medium (the right panels in Fig. 3) was detected to interfere the signal of the spiked analytes in the cell medium except the matrix-spiked EE2 (IS). Likewise, no background (or matrix) signal acquired from the non-treated cell lysate (the right panels in Fig. 4) was detected to interfere the signal of the analytes spiked in the cell lysate except the endogenous E1 and E2 present in the non-treated cell lysate (as indicated in Fig. 4).

3.2. Quantification methods

Some reports have used standard solutions in constructing the calibration curves for quantification [23]. Calibration curves established by using standard solutions, however, could suffer from some degrees of the matrix effect [27]. Thus, we decided to use the standards spiked in the non-treated cells for constructing the calibration curves to quantifying estrogens and EMs. There are two concerns regarding such calibration method. The first concern is that the spiked standards may conjugate with the matrix during the sample preparation process and lead to errors for quantification [28]. To prevent the conjugation, the spiked working standards were processed immediately after spiking. The data obtained with and without glucuronidase/sulfatase hydrolysis, which could break the conjugation, did not differ significantly. Furthermore, the recovery rate for the process including the spiking and extraction was determined to range from 90% to 126% with relative standard deviation ranging from 1% to 15% for the QC samples, which is within a common range of variations for method development. Thus, we confirmed that the quantification method using spiked estrogens and EMs standards in both the cell medium and the cell lysate will not cause significant errors due to matrix conjugation.

The second concern is the intrinsic estrogen and EMs present in non-treated cells which we also intended to quantify. There were indeed significant (S/N > 3) amounts of endogenous E1 and E2 detected in the non-treated cell lysate (Fig. 4). The endogenous E1 and E2 were also detected in the cell medium although they could not be viewed from Fig. 3 due to the large Y scale. In principle, standard addition will be the most suitable method for quantifying complex samples when blank background is difficult to find. Whereas, such quantification method needs a lot of labor to generate the data because multiple additions are required for each sample to be measured. Thus, we decided to use a calibration curve–standard addition hybrid method for quantifying estrogen and EMs for this study. For constructing the calibration curve, the signal of endogenous background (S_{back}) deduced from the non-treated cells was subtracted from the signal of the spiked standards (S_{std}) and the regression line was forced to zero intercept. Signals obtained from DMSO or E2-treated cells were used to determine their corresponding analyte concentrations based on the calculation from the constructed calibration curves. On the other hand, the concentration (C_{back}) of endogenous estrogens and EMs present in the non-treated cells was deduced by extrapolating the regression line to the minus axis value of the background signal (S_{back}). Since there were no previous studies that provided representative concentrations of all individual EM in the treated MCF-7 cell medium and the cell lysate, the calibration curves for quantifying

each EM in this study were constructed by one or two linear regression lines that cover a 10^3-fold concentration range with regression correlation coefficients ranging from 0.985 to 0.999.

3.3. Figure of merits

The limits of quantification (LOQs) were the lowest concentration in the calibration curve for each analyte and their accuracy and precision (Table 1) were studied by the QC samples prepared separately. From the data, the method has LOQs ranging from 6.24 pM (0.05 pg on column) to 9.77 nM (83 pg on column) with an accuracy ranging from 92.0% to 123.3% and a precision ranging from 1.2% to 9.6% ($n = 3$) for the cell medium; and LOQs ranging from 46.8 pM (0.38 pg on column) to 1.01 nM (8.3 pg on column) with an accuracy ranging from 71.7% to 109.0% and precision ranging from 3.6% to 10% ($n = 3$) for the cell lysate. For analytes which were not detected from the non-treated cells, the limits of detection (LODs) (Table 2) were determined based on the subtracted signals ($S - S_{back}$) to noise ratio of 3 ($(S - S_{back})/N = 3$). For E1 and E2 which were detected to be present in the non-treated cells (or blank cell medium and cell lysate), the LOD was determined based on the absolute signal to noise ratio of 3 ($S/N = 3$) (Table 2). Generally speaking, the LODs range from 4.10 pM (0.033 pg on column) to 110 pM (0.90 pg on column) for the cell medium and 18 pM (0.15 pg on column) to 300 pM (2.50 pg on column) for the cell lysate.

3.4. Metabolite profiling in endogenous MCF-7 cells

The extrapolation method described in the previous section was used to determine the endogenous estrogen or EMs concentration in non-treated cells. Since each 15-mL dish contained 1×10^6 cells, the obtained concentrations could be converted to fg/cell by multiplying these values (in nM) with a factor of 4.5 ($0.015\,L \times 300\,Da \times 10^{-6}$ cells $\times\ 10^6$ fg/cell). As shown in Table 3, under the non-treated condition, only trace amount of E1 (0.050 nM or 0.225 fg/cell in the medium; 0.076 nM or 0.342 fg/cell in the lysate) and E2 (0.150 nM or 0.675 fg/cell in the medium; 0.170 nM or 0.765 fg/cell in the lysate) were detected. We further investigated whether or not 10 μL of DMSO as the dissolving solvent will affect estrogens and EMs in the cells. Upon DMSO treatment, E1 and E2 concentration in the cell lysate increased to 7.31 nM (32.90 fg/cell) and 1.78 nM (8.01 fg/cell), respectively, while not much significant changes were detected in the cell medium (Table 3). In addition, 2-MeOE2 (medium: 0.015 nM or 0.068 fg/cell; lysate: 0.026 nM or 0.117 fg/cell), 4-MeOE2 (medium: 0.005 nM or 0.0225 fg/cell; lysate: 0.038 nM or 0.171 fg/cell), 2-OHE2 (medium: 0.150 nM or 0.675 fg/cell; lysate: 0.249 nM or 1.125 fg/cell), 4-OHE2 (medium: 0.250 nM or 1.125 fg/cell; lysate: 0.48 nM or 2.160 fg/cell) were also detected (Table 3) upon DMSO treatment but the rest EMs were not detected in either the medium or the lysate. From the data (Table 3), it is interesting to note that DMSO could induce slight increases in estrogens and their EMs. Whereas, these DMSO-induced EMs were majorly present in the cell lysate but only little amount were detected in the cell medium. Upon the treatment of 1 μM E2, the concentration of E2 in MCF-7 cells was substantially increased in the cell lysate (12.90 nM or 58.1 fg/cell) as well as in the cell medium (673.92 nM or 3033 fg/cell); E1 concentration was also substantially increased in the cell medium (178.74 nM or 804.33 fg/cell) but was decreased in the cell lysate (1.75 nM or 7.88 fg/cell). E2 significantly increased the amount of most EMs in the cell medium: E3 (1.50 nM or 6.75 fg/cell), 2-MeOE1 (0.14 nM or 0.63 fg/cell), 4-MeOE1 (0.69 nM or 3.11 fg/cell), 2-MeOE2 (1.13 nM or 5.09 fg/cell), 4-MeOE2 (5.66 nM or 25.5 fg/cell), 2-OHE1 (0.76 nM or 3.42 fg/cell), 4-OHE1 (0.59 nM or 2.66 fg/cell), 2-OHE2 (1.34 nM or 6.03 fg/cell), and 4-OHE2 (1.79 nM or 8.06 fg/cell). Whereas, less significant changes were detected from the cell lysate compared to DMSO treatment (Table 3). It is particularly notable that 16α-OHE1 was the only EM that was not detected in either the cell medium or the cell lysate but its metabolite E3 (1.50 nM or 6.76 fg/cell) was detected to be present in the cell medium upon E2 treatment.

It is generally believed that most of the responsible enzymes such as 17β-hydroxysteroid dehydrogenases (17β-HSD) that converts 16α-OHE1 and E1 to E3 and E2, respectively; cytochrome P450 hydroxylase [29,30] that converts E2/E1 to 4-OHE2/E1, 2-OHE2/E1, or 16α-OHE1; and COMT [31] that converts 4-OHE2/E1 and 2-OHE2/E1 to 4-MeOE2/E1 and 2-MeOE2/E1, respectively, are present in the intracellular compartment. Thus, we postulate that most of the EMs detected in the cell medium were produced in the intracellular compartment and then secreted to the extracellular medium within 24 h of E2 treatment and 16α-OHE1 produced from E2 metabolism was quickly converted to E3 before it was secreted to the cell medium [32]. Moreover, the data also implied that DMSO exhibited slight estrogen effect which should raise attentions when DMSO was used as the solvent for drugs. This observation is consistent with the report that DMSO could induce significant increases in estrogen receptor α, estrogen receptor β, vitellogenin and *zona radiata*-protein genes in a time-dependent manner as observed from real-time polymerase chain reaction and indirect ELISA analysis [33]. Our data revealed the DMSO-induced EMs and further indicated that the secretion speed of EMs induced by DMSO could be relatively slow compared to those induced by E2 in a cancer cell.

4. Conclusion

We reported a simple yet reliable LC–MS/MS SRM method for detecting endogenous estrogens and EMs in the intracellular and extracellular compartment of MCF-7 cells using EA extraction and dansyl chloride derivatization. To the best of our knowledge, this is the first report to quantitatively measure estrogens and EMs present in both the extra- and intra-cellular compartment of a cell. Although the current method was developed for detecting free estrogen and EMs, it can be easily modified to detect the conjugated forms. Furthermore, our data indicated that E2 could be quickly metabolized to form secreted EMs and DMSO could also induce slight EMs, which are interesting and worth further investigations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.04.020.

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