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Extractive ethoxycarbonylation in high-temperature gas chromatography–mass spectrometry based analysis of serum estrogens

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ABSTRACT

A comprehensive gas chromatography–mass spectrometry (GC–MS)-based profiling was developed as a practical assay for quantification of 18 endogenous estrogens in serum samples. The present GC–MS method was conducted with the two-phase extractive ethoxycarbonlyation (EOC) of the phenolic hydroxy groups of estrogen with ethyl chlorformate combined with the non-polar n-hexane extraction. The subsequent perfluoroacylation of aliphatic hydroxy groups with pentafluoropropionyl anhydride (PFPA) was conducted. The serum samples were separated through a high temperature GC column (MXT-1) within an 8-min run and analyzed in selected-ion monitoring mode with good chromatographic properties for 18 estrogens as their EOC-PFP derivatives. The limit of quantification (LOQ) was 0.025–0.10 ng/mL for most estrogens analyzed except for E3 and 2-OH-E3 (0.5 ng/mL each). The devised method was found to be linear over a 10^3-fold concentration range with a correlation coefficient ($r^2 > 0.992$), whereas the precision (% CV) and accuracy (% bias) ranged from 3.1 to 16.3% and from 93.5 to 111.1%, respectively. Decreased 2-methoxy-17β-estradiol levels were confirmed in patients with preeclampsia than healthy pregnant women. This technique can be used for a clinical diagnosis as well as understanding the pathogenesis in estrogen-related disorders.

1. Introduction

Endogenous estrogens play an important role in the pathophysiological mechanisms of women's cancers [1–4] and reproductive progress [5–7]. Recently, two studies reported the critical role of the estrogen metabolism in preeclampsia referred to as pregnancy-induced hypertension [8,9], which is a systemic disorder of pregnancy characterized by a widespread maternal endothelial dysfunction that is a leading cause of maternal and fetal morbidity and mortality worldwide [10,11].

Estrogens in women are biosynthesized in the ovaries and are metabolized primarily through NADPH-dependent hydroxylation [12]. The hydroxylation of estrogens on either the A-ring or D-ring is catalyzed by various cytochrome P450 enzyme isoforms and results in the formation of hydroxy and keto metabolites (Fig. 1). Hydroxylation at the C2 position on the A-ring has a larger extent than the C4 position [13], and 2- and 4-hydroxy estrogens are further metabolized by catechol-O-methyltransferase (COMT) to 2-, 3-, and 4-methoxy estrogens [14]. Hydroxylation at the 16α position of the D-ring produces 16α-hydroxyestrone, which can be metabolized further to estriol, 17-epiestriol, 16-ketoestradiol, and 16-epiestriol [15]. According to the carcinogenic effect of breast cancer [1,16–18], catechol estrogens form quinones that react with DNA and form both stable and depurinating DNA adducts. The mitogenic and antiaptotic effects of estrone, estradiol and some of their hydroxylated metabolites could be evaluated.

The estrogen metabolites are present in serum as a free-form, non-specific binding protein form and acid conjugates [19]. The concentration of conjugated estrogens in the serum is 2–3 times higher than that of the free-forms [20]. The circulating levels of estrogen metabolite in serum have been measured by a radio- or enzyme immunoassay (RIA or EIA) [21,22], and gas or liquid chromatography with mass spectrometry-based methods (GC–MS [23–25] or LC–MS [26–28]). Although the immunoassays are sensitive, they are hampered by overestimations caused by cross-reactions [29–31]. In recent, MS-based profiling is a proven technique with high sensitivity and selectivity in estrogens analysis combined with chemical derivatization for the phenolic hydroxy groups of estrogens as follows: (1) pentafluorobenzyl (PFB) [25],

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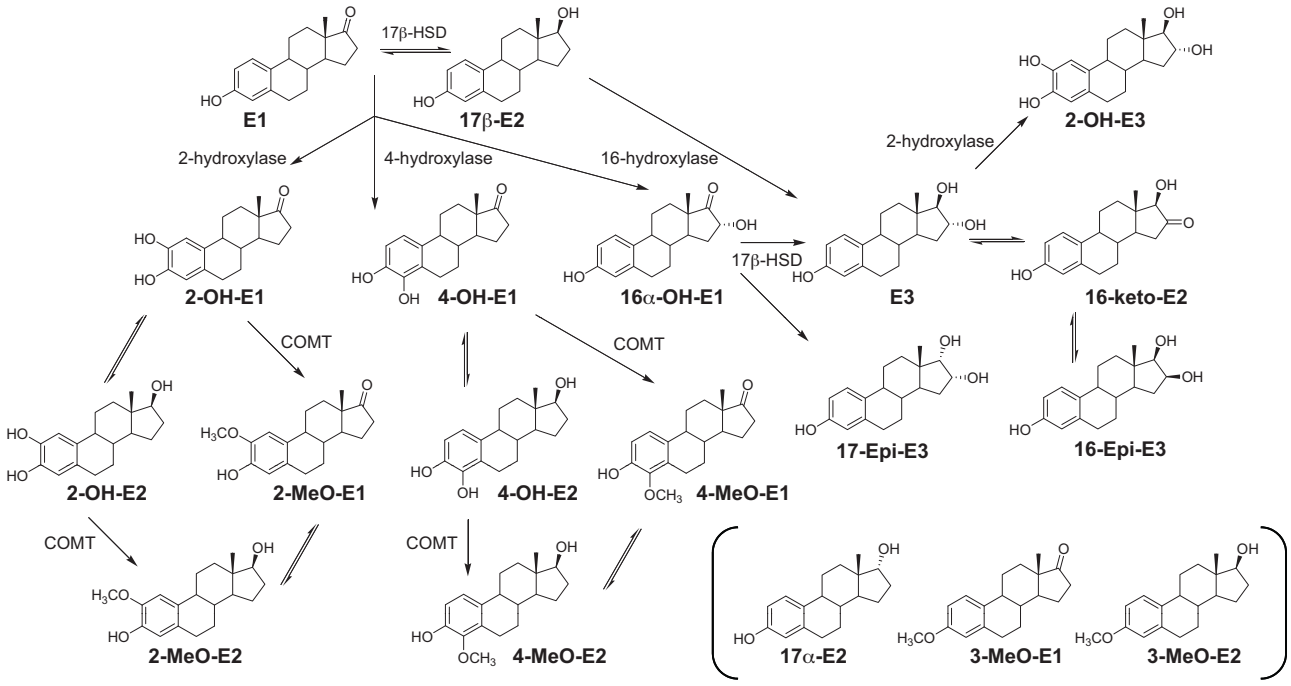

Fig. 1. Proposed estrogen metabolism in humans. Enzymes involved in the estrogen metabolism: (1) 17β-HSD, 17β-hydroxysteroid dehydrogenase; (2) 2-, 4-, or 16α-hydroxylase; (3) COMT, catechol-O-methyltransferase. Among them, the metabolic pathway of three estrogen metabolites, 17α-E2, 3-MeO-E1 and 3-MeO-E2, is unknown. See Table 1 for full names of the endogenous estrogen metabolites.

(2) dansyl [20,26] and (3) N-methyl-2-pyridyl [19] derivatization coupled with LC–MS analyses. They resulted in low pg/mL-level sensitivity but the methods required time-consuming derivatization [27] or a long analytical run [20]. In high-resolution GC–MS analysis, estrogens should be blocked by the active hydrogens in both phenolic and aliphatic hydroxyl groups. For this purpose, perfluoroacylation and trimethylsilylation were performed [23–25]. As a different approach, alkoxycarbonylation, which is conducted with highly reacting agents for amino-, thiol-, imidazole- or phenolic hydroxyl groups, could be suitable for direct reactions in aqueous media [32–35]. This direct-derivatization to make analytes extractable by non-polar organic solvents can be achieved at the same time. Moreover, it is also rapid and reproducible with an excellent purification [32,34,35].

This study describes an optimized two-phase extractive ethoxycarbonylation (EOC) with ethylchloroformate (ECF) for the comprehensive analytical method of 18 endogenous estrogens including catechol estrogens to overcome their high polarity and instability by GC–MS-based analysis. Subsequent perfluoroacylation with pentafluoropropionyl anhydride (PFPA) were combined. The resulting EOC-PFP estrogen metabolites were separated through a high temperature GC column and results in better detectability with a short analytical run compared to a fused-silica GC column [36,37]. This method was used to quantify altered estrogens from patients with preeclampsia and healthy pregnant women because the estrogen metabolism plays a role in the pathophysiology of preeclampsia.

2. Experimental

2.1. Chemicals and reagents

The 19 endogenous estrogens including estrone (E1), 17β-estradiol (17β-E2), estriol (E3), 2-hydroxyestrone (2-OH-E1), 2-hydroxy-17β-estradiol (2-OH-E2), 4-hydroxyestrone (4-OH-E1), 4-hydroxy-17β-estradiol (4-OH-E2), 2-methoxyestrone (2-MeO-E1), 2-methoxy-17β-estradiol (2-MeO-E2), 3-methoxyestrone (3-MeO-E1), 3-methoxy-17β-estradiol (3-MeO-E2), 4-methoxyestrone (4-MeO-E1), 4-methoxy-17β-estradiol (4-MeO-E2), 17-epiestriol (17-epi-E3), 16-epiestriol (16-epi-E3), 16α-hydroxyestrone (16α-OH-E1), 16-keto-17β-estradiol (16-keto-E2), 2-hydroxyestriol (2-OH-E3), and 17α-estradiol (17α-E2) were obtained from Steraloids (Newport, RI, USA). The deuterium-labeled internal standard (IS), 2,4,16,16-d_4-17β-estradiol (d_4-E2, isotopic purity ≥ %) was purchased from C/D/N isotopes (Pointe-Claire, Quebec, Canada).

In solid-phase extraction (SPE), Oasis HLB (3 cm^3, 60 mg; Waters, Milford, MA, USA) preconditioned with 2 mL of methanol followed by 2 mL of deionized water was used. Sodium acetate (reagent grade), acetic acid (glacial, 99.99+%) and L-ascorbic acid (reagent grade) were obtained from Sigma (St. Louis, MO, USA). A solution of β-glucuronidase/arylsulfatase from *Helix pomatia* was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Anhydrous potassium carbonate anhydrous (K_2CO_3), triethylamine (TEA) and ethylchloroformate (ECF) were obtained from J. T. Baker (Phillipsburg, NJ), Sigma, and Daejung Chemicals Co. (Shiheung, Gyonggi, Korea), respectively. The acylation reagent, pentafluropropionic anhydride (PFPA) was obtained from Sigma. All organic solvents used as the analytical and HPLC grades were purchased from Burdick & Jackson (Muskegan, MI, USA). Deionized water was prepared using a Milli-Q purification system (Millipore, Billerica, MA, USA).

2.2. Standard solutions and quality-control samples

Each stock solution of all reference standards including internal standard d_4-E2 was prepared at a concentration of 1 mg/mL in methanol, whereas 2-OH-E1, 2-OH-E2, 4-OH-E1, 4-OH-E2 and 2-OH-E3 were dissolved with L-ascorbic acid containing methanol (1 mg/mL) to prevent oxidation. The working solutions were diluted with a methanolic ascorbic acid solution at various concentrations in the range of 0.001–10 μg/mL. All standard solutions

were stable for a minimum of 3 months at 4 °C in a teflon-sealed amber-glass vial. Commercially available steroid-free serum (Scipac; Sittingbourne, UK) was used for calibration and quality-control (QC) purposes. After checking that the endogenous estrogens were not presented by GC–MS, the calibration samples were made up at 15 different concentrations, which range from 0.025 to 1000 ng/mL. The quality control samples were also prepared at 7 different concentrations (0.1, 0.5, 1, 2, 5, 20 and 100 ng/mL) with steroid-free serum depending on the sensitivity and reference values of the serum estrogens in pregnant women.

2.3. Subjects and sample collection

The serum samples were collected from 17 healthy pregnant women (age: 30.5 ± 4.2 yr) and 17 severe preeclampsia patients (age: 31.6 ± 3.2 yr) in the Department of Obstetrics and Gynecology at Inje University (Busan, Korea). All samples were from gestational age 22–35 weeks. The characteristics in age (yr), gestational age (wk), and BMI (kg/m^2) were similar in the two groups. According to the American College of Obstetricians and Gynecologists recommendations [14], preeclampsia was defined as new-onset hypertension after 20 weeks gestation, such that the systolic blood pressure of ≥140 mm Hg, diastolic blood pressure of ≥140 mm Hg, or both were observed on two occasions ≥6 h apart, with significant proteinuria ≥300 mg/24 h. Severe preeclampsia involves a blood pressure > 160 mm Hg or higher systolic or 110 mm Hg or higher diastolic on two occasions at least 6 h apart in woman on bed rest and with proteinuria over 5 g in a 24 h urine collection [14,15]. All samples were stored at −20 °C until needed.

2.4. Sample preparation

The serum sample (0.4 mL) was diluted with 2.6 mL acetate buffer (0.2 M, pH 5.2) and 100 μL of 0.2% aqueous L-ascorbic acid and 20 μL of the internal standard, d_4-E2 (200 ng/mL) was then added. The samples were extracted with Oasis HLB^{TM} SPE cartridges placed in a device fitted with a small peristaltic pump and operated at a low flow rate (<1 mL/min). After loading the sample on a cartridge, each was washed with 2 mL water and eluted twice with 2 mL of methanol. The combined methanol was evaporated under a nitrogen stream and added to 1 mL of 0.2 M acetate buffer (pH 5.2), 100 μL of aqueous 0.2% L-ascorbic acid, and 50 μL of β-glucuronidase/arylsulfatase. After incubation at 55 °C for 3 h, the solution was adjusted to pH 8 with a 5% K_2CO_3 solution and 30 μL of TEA and 50 μL of ECF were added. After vortexing for 30 s, the sample was extracted twice with a 2.5 mL non-polar solvent n-hexane. The organic solvent was evaporated by an N_2 evaporator at 40 °C and further dried in a vacuum desiccator over P_2O_5–KOH for at least 30 min. Finally, the dried residue was derivatized with 20 μL of PFPA in 100 μL of n-hexane at 50 °C for 30 min, and evaporated using an N_2 evaporator. The resulting product reconstituted with 30 μL of n-hexane (2 μL) was injected for GC–MS analysis in selected-ion monitoring (SIM) mode.

2.5. Instrumental conditions

GC–MS was performed with an Agilent 6890 Plus gas chromatograph interfaced with a single-quadrupole Agilent 5975 MSD. The electron energy was 70 eV and the ion source temperature was 230 °C. Each sample (2 μL) was injected in split mode (8:1) at 280 °C and separated through a MXT-1 (30 m × 0.25 mm I.D., 0.25 μm film thickness, Silcosteel-treated stainless steel) cross-linked dimethyl polysiloxane capillary column. The oven temperature was initially at 270 °C. The temperature was ramped to 300 °C at 6 °C/min and then finally increased to 330 °C using a 10 °C/min ramping program. The column head pressure of helium as a carrier gas was set to 151.7 kPa. For quantitative analysis, characteristic ions of each estrogen were determined as their EOC-PFP derivatives in SIM mode with a dwell time of 100 ms. Peak identification was achieved by comparing the retention times and matching the height ratios of the characteristic ions (Table 1).

2.6. Method validation

The QC samples containing 18 estrogen metabolites were used over the course of 3 months and quantification was performed using the peak height ratios relative to that of the IS. Least-squares regression analysis was performed on the peak height ratios with increasing amounts for calibration. The limit of detection (LOD) and quantification (LOQ) were defined as the lowest concentration with a higher signal-to-noise (S/N) ratio than 3 for LOD and 10 for LOQ. The precision expressed as the coefficient of variation (% CV) and the accuracy as the percentage relative error (% bias) of the method were determined using QC samples at three different concentrations (low: 0.1, 0.5, 1 or 2 ng/mL, medium: 2, 5 or 20 ng/mL, and high: 20 or 100 ng/mL) depending on the sensitivity and reference values of the serum estrogens in pregnant women. For the within-day repeatability, 5 replicates were analyzed, whereas the reproducibility was measured from the samples run over 5 different days.

The overall recovery was established using QC samples at three concentrations in triplicate for each estrogen by adding known amounts of mixed working solutions to the steroid-free serum samples. Absolute recovery was calculated by comparing the analytical results of the samples through the whole sample preparation with those of the standard samples without SPE and enzymatic hydrolysis that represent 100% recovery.

The stability of the analyte during sample collection and handling was also evaluated. The stability was measured by comparing the results of the samples analyzed before and after being exposed to the conditions for a stability assessment at three different concentrations in triplicate. (1) The stability of the standard solutions was tested by standing at room temperature for 6 h over the time needed for sample preparation. (2) Freeze and thaw stability was determined after three freeze and thaw cycles. After storing three aliquots of QC samples at −20 °C for 24 h, the samples were thawed at room temperature. When thawed completely, the samples were refrozen for 12 h under the same conditions, which were repeated three times. (3) The short-term temperature stability was evaluated by thawing the QC samples at ambient temperature and leaving them to stand at this temperature for 6 h. (4) The post-preparative stability was evaluated by a reinjection of the prepared samples after 6 h (after one batch analysis of validation samples) and 30 h (after one day from being put in the sample tray of the auto-injector).

3. Results and discussion

3.1. GC–MS characteristics of EOC-PFP derivatives

Estrogen metabolites have one or two phenolic hydroxy groups as well as other polar groups, such as aliphatic hydroxy or ketone [12,13] (Fig. 1). When high-resolution capillary GC–MS is used for the estrogen analysis [23–25], it is essential to block the active hydrogens in polar functional groups. Although the trimethylsilylation (TMS) is commonly used in GC–MS based estrogen analysis [23,24], it is not enough to reach sensitivity for the quantification of estrogen metabolites in serum samples at levels in the low sub-ng/mL range. To enhance the specificity and sensitivity, a comprehensive derivatization for estrogen analysis was conducted with the extractive EOC with ECF in the aqueous phase, and was applied

Table 1
GC–MS information on the quantitative analysis of 19 estrogens as their EOC-PFP derivatives.

Compounds (trivial name)	Abbreviation	Molecular ion	Characteristic ion[a]	Quantitative ion[b]	Retention time (min)
3-Methoxy-17β-estradiol	3-MeO-E2	432	432, 404	432 $[M]^+$	3.00
3-Methoxyestrone	3-MeO-E1	284	284, 227	284 $[M]^+$	3.37
17α-Estradiol	17α-E2	490	490, 446, 418	418 $[M-72]^+$	4.04
Estriol	E3	652	652, 608, 580	580 $[M-72]^+$	4.10
17β-estradiol	17β-E2	490	490, 446, 418	418 $[M-72]^+$	4.34
17-Epiestriol	17-epi-E3	652	652, 608, 580	580 $[M-72]^+$	4.42
16-Epiestriol	16-epi-E3	652	652, 608, 580	580 $[M-72]^+$	4.81
16α-Hydroxyestrone	16α-OH-E1	504	504, 460, 432	432 $[M-72]^+$	4.81
4-Methoxy-17β-estradiol	4-MeO-E2	520	520, 476, 448	448 $[M-72]^+$	4.84
Estrone	E1	342	342, 298, 270	270 $[M-72]^+$	4.90
2-Methoxy-17β-estradiol	2-MeO-E2	520	520, 476, 448	448 $[M-72]^+$	5.11
16-Keto-17β-estradiol	16-keto-E2	504	504, 460, 432	432 $[M-72]^+$	5.21
4-Methoxyestrone[c]	4-MeO-E1	372	372, 328, 300	300 $[M-72]^+$	5.47
2-Methoxyestrone	2-MeO-E1	372	372, 328, 300	300 $[M-72]^+$	5.73
2-Hydroxyestriol	2-OH-E3	740	740, 696, 652, 624, 596	596 $[M-72-72]^+$	6.23
4-Hydroxy-17β-estradiol	4-OH-E2	578	578, 534, 506, 490, 462, 434	434 $[M-72-72]^+$	6.35
2-Hydroxy-17β-estradiol	2-OH-E2	578	578, 534, 506, 490, 462, 434	434 $[M-72-72]^+$	6.64
4-Hydroxyestrone	4-OH-E1	430	430, 386, 358, 342, 314, 286	286 $[M-72-72]^+$	7.06
2-Hydroxyestrone	2-OH-E1	430	430, 386, 358, 342, 314, 286	286 $[M-72-72]^+$	7.31

[a] All estrogens were derivatized with pentafluropropionic anhydride (PFPA) for the hydroxy groups after ethoxycarbonylation (EOC) with ethyl chloromate for the phenolic hydroxy groups of estrogens in an aqueous buffer, except for the estrogens containing no hydroxy groups. All ions are given as within 30% of the base peak.
[b] Base peaks were chosen as the quantitative ions of estrogens.
[c] 4-MeO-E1 was not evaluated because of the high background signals from the serum samples analyzed.

successfully to protect the active phenolic hydroxy hydrogens in estrogen molecules as direct-derivatization techniques [32]. To improve the detectability with good GC–MS properties, perfluoroacylation with PFPA for protection of the remaining aliphatic hydroxy groups was conducted as subsequent derivatization. The chromatographic separation of the 19 estrogens as their EOC-PFP derivatives was achieved by a high-temperature GC column with excellent peak shapes and higher responses within an 8-min run (Fig. 2). In general, a high-temperature GC column shows better chromatographic properties in an analysis of low volatile lipophilic compounds [37–39].

Using EOC-PFP derivatization, 16α-OH-E1 could separate from 16-keto-E2, whereas they were co-eluted in TMS and EOC-TMS derivatizations [23]. Both compounds had the same molecular weight and similar mass fragments. In general, 16α-OH-E1 stimulates cell proliferation in breast cancer cell lines and shows estrogenic and genotoxic potential in oxidative stress induced biological actions [2]. Its accurate quantification is very important and EOC-PFP derivatization gave selective and sensitive detection in serum samples. Although three estrogens (e.g., 16-epi-E3, 16α-OH-E1, and 4-MeO-E2) were co-eluted in the present method, they were differentiated by SIM because of their different characteristic ions at *m*/*z* 580, 432, and 448, respectively (Table 1).

An interpretation of the mass spectrometric patterns of estrogen metabolites allowed the differentiation of mono- and di-EOC-PFPA derivatives among the 19 estrogens (Table 1). The base peak of all mono-EOC-PFPA derivatives was $[M-72]^+$ ion corresponding to the loss of the EtOCO-group, whereas minor peaks were formed at $[M]^+$ and $[M-44; M\text{-}OEt]^+$ ions. For five di-EOC-PFPA derivatives of catechol estrogens, the loss of $[M-72-72; M\text{-}2EtOCO-]^+$ ions was formed as a base peak, whereas $[M-72-44]^+$, $[M-44-44]^+$, $[M-72]^+$, $[M-44]^+$, and $[M]^+$ ions were observed as minor peaks. In addition, 3-MeO-E1 and 3-MeO-E2 without phenolic hydroxy groups generated the molecular ion as the base peak at *m*/*z* 284 and at *m*/*z* 432, respectively.

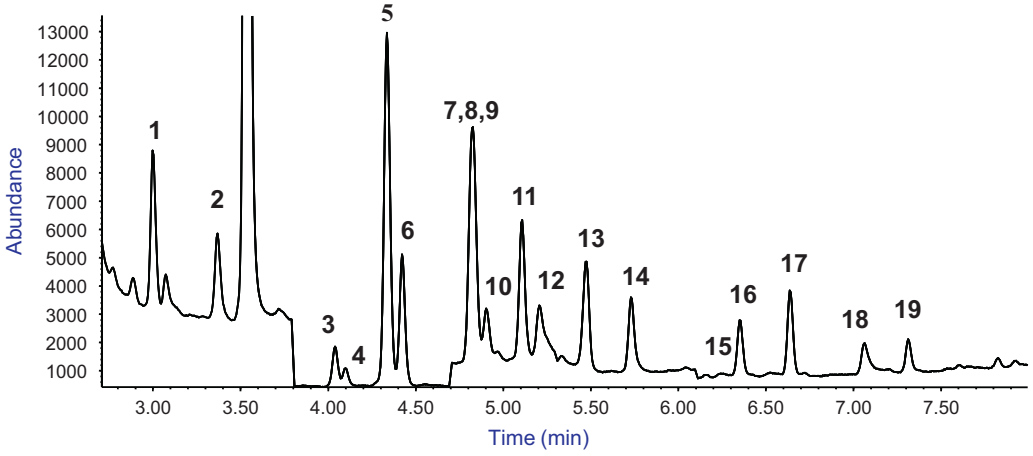

Fig. 2. Total-ion chromatogram of the 19 estrogens as their EOC-PFP derivatives in GC–SIM/MS analysis. The 19 estrogens (10 ng/mL each) were spiked into the steroid-low serum and prepared for quantitative analysis. Peak: 1. 3-MeO-E2; 2. 3-MeO-E1; 3. 17α-E2; 4. E3; 5. 17β-E2; 6. 17-epi-E3; 7. 16α-OH-E1; 8. 16-epi-E3; 9. 4-MeO-E2; 10. E1; 11. 2-MeO-E2; 12. 16-keto-E2; 13. 4-MeO-E1; 14. 2-MeO-E1; 15. 2-OH-E3; 16. 4-OH-E2; 17. 2-OH-E2; 18. 4-OH-E1; 19. 2-OH-E1. The sample was separated through a thermally stable MXT-1 capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness). The oven temperature was initially at 270 °C and ramped to 300 °C at 6 °C/min and then finally increased to 330 °C at 10 °C/min.

3.2. Sample preparation

In this study, ethoxycarbonylation [33–35], which is applicable to a range of compounds containing amino- or phenolic hydroxy groups of steroids, was introduced and optimized for the analysis of 19 endogenous estrogens as a simple and fast sample preparation for GC–MS analysis. After ethoxycarbonylation, n-hexane was used as an optimal extraction solvent to compromise high extraction efficiency and removing the matrix interference. Extraction with nonpolar solvents, such as n-hexane, under the consideration of increasing lipophilicity of analytes after ethoxycarbonylation is quite effective in removing disturbing polar substances in biological samples [34,35].

However, a disadvantage of this assay is that the background noise co-elutes with 4-MeO-E1 in serum samples, even in the commercial carbon-stripping steroid-free serum (Supplementary Fig. S1). Therefore, the SPE procedure was tested to remove the sample complexity, particularly the washing steps performed using 5% methanol with different fraction volumes (1–7 mL) but the background problem still remained. On the other hand, the commercial steroid-low serum made by stripping with activated carbon do not also remove completely some of the compounds bound to serum lipoproteins (e.g., sterols) [40]. Therefore, some studies have used some type of artificial or surrogate matrix, such as phosphate-buffered saline (PBS), or diluted bovine (BSA) or human serum albumin [40,41]. In this study, although a quantitative determination of 4-MeO-E1 at low-concentrations was difficult, commercial steroid-free serum was chosen as a suitable matrix for the standard calibration of 18 estrogens except for 4-MeO-E1.

3.3. Method validation

The LOQs of the EOC-PFP derivatives with the E1-type moiety were 0.10–0.5 ng/mL, whereas those of most estrogens with the E2- or E3-type moiety ranged from 0.025 to 0.10 ng/mL, except for E3 and 2-OH-E3 (0.5 ng/mL each), which might be due to the low recovery obtained from n-hexane extraction after ethoxycarbonylation (Table 2). Most estradiol- or estriol-type compounds that contain aliphatic hydroxy groups at the 16 and/or 17-position on the D-ring showed a significantly higher S/N ratio than the estrone-type compounds, which have no aliphatic hydroxy groups for the introduction of subsequent derivatization with PFPA. The overall recoveries of the EOC-PFP derivatives ranged from 71.1 to 110.7%, with the exception of 31.4% for E3 and 25.1% for 2-OH-E3.

An effective clinical assay will have the ability to measure specific analytes accurately over a wide range of concentrations, particularly for pregnant women. With this method, the calibration curve consisted of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and 15 samples covering from the LOQ to the expected range in the sample. The devised method was linear over a 10^3-fold concentration range with a correlation coefficient ($r^2 > 0.992$) in the range from LOQ to the expected range in the sample of pregnant women. The precision and accuracy were determined by analyzing the QC samples acquired for intra- and interday assays at 3 different levels: low (0.1, 0.5, 1 or 2 ng/mL), medium (2, 5 or 20 ng/mL) and high (20 or 100 ng/mL) concentrations. The intra-day ($n = 5$) precision (expressed as % CV) ranged from 3.0 to 15.3%, whereas the accuracy (expressed as % bias) ranged from 92.6 to 115.7%, and the inter-day ($n = 5$) precision (% CV) and accuracy (% bias) ranged from 3.1 to 17.3% and from 93.3 to 106.5%, respectively (Table 2).

The stability tests were evaluated for the reliable quantification of estrogens, including standard solution storage, short-term storage (bench-top, room temperature), freeze and thaw cycles, and analytical process, as described in the Experimental section (Table 3). Both stock solutions and QC samples were prepared freshly and L-ascorbic acid (1 mg/mL) was added to prevent the oxidative degradation of catechol estrogens. The standard solutions were stable not only at −20 °C for three months but also at room temperature for 6 h. The short-term stability, which was tested by thawing the QC samples at 25 °C and leaving them to stand for 6 h, showed no significant changes (≤15% RSD) in concentration under the conditions tested, only the catechol estrogens were likely to be more or less unstable compared to the other estrogens. An evaluation of the freeze/thaw stability was performed by measuring the concentration of the aliquot not subjected to the freeze/thaw cycles as a reference. The stability of estrogens was also demonstrated in the serum samples subjected to three freeze/thaw cycles. The overall differences between the cycles were not significant within 15% deviation in most estrogens. Repeated freezing and thawing cycles did not appear to affect the concentrations of most estrogens except for catechol estrogens (≤21% RSD). The post-preparative stability under the analysis conditions including the autosampler conditions for the expected maximum time of an analytical run was performed to determine if the analytical run could be reanalyzed in the case of instrumental failure. The results showed that the EOC-PFP derivatives of estrogens were quite stable when the prepared samples were injected after one batch from being placed in the sample-tray (≤9.0% RSD).

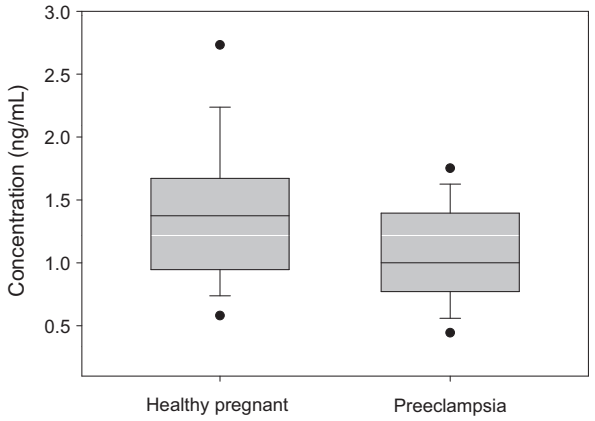

Fig. 3. Comparison of the serum 2-MeO-E2 levels in preeclampsia patients ($n = 17$; 1.0 ± 0.4 ng/mL) and healthy pregnant women ($n = 17$; 1.4 ± 0.5 ng/mL). The 2-MeO-E2 concentrations were significantly lower in the preeclampsia patients ($P < 0.05$).

3.4. Application into the preeclampsia samples

The validated method was used to measure the levels of 18 endogenous estrogens in the serum samples obtained from preeclampsia patients and age-matched healthy pregnant women. Among the 18 estrogens monitored, 13 estrogens including E1, 17β-E2, E3, 17-epi-E3, 16-epi-E3, 16α-OH-E1, 16-keto-E2, 2-OH-E1, 2-OH-E2, 2-OH-E3, 4-OH-E2, 2-MeO-E1, and 2-MeO-E2 were detected quantitatively. Among the estrogens detected, E3 was at the highest concentration, followed by E1, 17β-E2, 16α-OH-E1, and 16-keto-E2. The levels of 2-MeO-E2 were lower in the preeclampsia patients than in the healthy pregnant (1.0 ± 0.4 ng/mL vs. 1.4 ± 0.5 ng/mL; $P < 0.05$; Fig. 3). This result is in accordance with a previous report, which found that the 2-MeO-E2 levels in preeclmapsia patients ($P < 0.05$) are lower than in control subjects [8]. 2-MeO-E2 is generated in the placenta and protects against generalized endothelia dysfunction. The involvement of other estrogens in preeclmapsia also needs to be addressed in future clinical studies.

Table 2
Validation results of the overall method with intra- and inter-day assays.

Compounds (abbreviation)	LOD[a] (ng/mL)	LOQ[b] (ng/mL)	Recovery[c] (%)	Linearity[d] (r^2)	Intra-day ($n = 5$)		Inter-day ($n = 5$)	
					CV[e] (%)	Accuracy[e] (%)	CV (%)	Accuracy (%)
E1	0.10	0.25	89.0	0.9992	3.8	104.8	5.7	98.8
17β-E2	0.01	0.03	95.1	0.9998	3.3	94.2	3.9	97.4
E3	0.10	0.50	31.4	0.9972	8.2	103.8	14.5	101.5
2-OH-E1	0.10	0.25	99.8	0.9954	3.2	92.6	9.2	93.3
2-OH-E2	0.02	0.10	92.8	0.9964	7.7	96.1	6.4	99.8
4-OH-E1	0.10	0.25	105.2	0.9939	6.8	104.2	9.6	98.4
4-OH-E2	0.02	0.10	83.2	0.9958	14.7	101.8	9.2	102.7
2-MeO-E1	0.05	0.10	96.0	0.9968	12.4	92.7	7.4	94.1
2-MeO-E2	0.05	0.10	82.6	0.9962	5.3	99.8	3.1	102.9
3-MeO-E1	0.10	0.25	90.5	0.9916	7.8	106.1	6.8	102.5
3-MeO-E2	0.05	0.10	99.7	0.9968	3.0	103.1	4.8	101.9
4-MeO-E2	0.02	0.05	101.6	0.9992	4.8	106.3	4.3	103.0
17-epi-E3	0.02	0.05	110.7	0.9982	3.9	112.8	8.3	104.3
16-epi-E3	0.05	0.10	76.2	0.9980	6.4	109.7	10.5	106.2
17α-E2	0.05	0.10	106.2	0.9994	8.4	101.4	4.1	99.5
16α-OH-E1	0.05	0.10	98.5	0.9966	11.7	104.8	12.4	96.8
16-keto-E2	0.10	0.25	71.1	0.9984	10.0	105.5	12.9	102.5
2-OH-E3	0.25	0.50	25.1	0.9954	15.3	115.7	17.3	106.5

[a] The limit of detection was measured at S/N ratio > 3.
[b] Limit of quantification was measured at a S/N ratio > 10.
[c] Absolute recoveries were calculated by comparing the peak height ratios of the samples using the described method versus those of their nonextracted counterparts from three different QC concentrations in 5 replicates.
[d] Calibration ranges were from the LOQ level to expected concentrations in pregnant women for each analyte and linearity was measured as the mean levels of data through 5 different days.
[e] The precision and accuracy were expressed as the mean values of data obtained from three QC samples through intra- and inter-day assays.

Table 3
Results of stability tests for the working solution, short-term storage, freeze–thaw cycles and post-preparations.

Compounds (abbreviation)	Working solution[a]		Short-term storage[b]		Freeze and thaw cycles[c]		Post-preparative stability[d]			
							Reinjection after 6 h		Reinjection after 30 h	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
E1	94.3	4.7	90.4	12.0	96.4	10.1	81.6	3.2	97.9	10.2
17β-E2	104.3	4.5	108.1	5.0	99.6	5.7	97.0	3.0	104.2	4.7
E3	102.5	4.6	91.8	14.1	98.8	12.3	102.6	2.3	103.5	5.5
2-OH-E1	89.0	4.8	58.7	16.7	57.7	19.7	103.7	5.4	65.5	37.8
2-OH-E2	97.9	4.2	90.5	16.4	108.8	17.4	99.7	3.0	93.7	16.6
4-OH-E1	87.2	1.5	53.4	9.4	56.7	19.1	95.4	4.3	70.5	22.9
4-OH-E2	98.5	5.4	77.3	18.8	109.6	21.0	100.7	2.9	93.6	16.9
2-MeO-E1	91.1	4.7	82.2	14.0	88.3	11.0	81.9	4.6	96.6	10.0
2-MeO-E2	102.9	4.3	108.2	6.8	103.5	8.0	101.5	1.1	97.1	7.5
3-MeO-E1	100.2	5.0	90.3	10.8	94.3	16.3	90.6	9.0	100.1	10.0
3-MeO-E2	108.2	6.3	101.2	3.4	101.8	5.5	100.9	3.3	102.1	1.7
4-MeO-E2	103.3	7.0	106.5	3.3	101.9	5.1	100.4	1.2	98.3	5.3
17-epi-E3	102.6	7.4	67.9	19.4	74.5	29.1	103.1	1.9	104.1	4.3
16-epi-E3	99.7	7.4	78.5	13.5	99.0	16.6	104.3	2.2	101.8	7.0
17α-E2	102.6	4.9	109.1	6.4	101.6	6.3	96.2	2.9	102.0	6.5
16α-OH-E1	99.0	0.6	90.4	11.9	93.0	8.0	88.2	4.1	77.8	4.6
16-keto-E2	96.0	5.3	66.6	8.4	90.0	10.1	98.0	2.5	84.5	6.3
2-OH-E3	100.1	5.6	83.6	14.8	105.2	19.1	102.5	5.0	95.0	14.8

[a] The working solution stability was calculated by comparing the concentrations of the samples using a fresh working solution versus those of the samples employed after leaving them to stand for 6 h at room temperature.
[b] The short-term stability was evaluated by thawing the QC samples at ambient temperature and then leaving them to standing at this temperature for 6 h.
[c] The freeze and thaw stability was determined after three freeze–thaw cycle. After storing three aliquots of QC samples at −20 °C for 24 h, these samples were thawed at room temperature. When completely thawed, the samples were refrozen for 12 h and then these processes were repeated three times.
[d] The post-preparative stability was evaluated by the reinjection of prepared samples after 6 h (after one batch analysis of validation samples) and 30 h (after one day from being put in the sample-tray in auto-injector). All stability was tested by comparing the results of samples analyzed before and after being exposed to the conditions for stability assessment at three different concentrations in triplicates.

4. Conclusions

A comprehensive GC–MS method was developed as a practical assay to quantify 18 endogenous estrogens in a serum sample. The devised technique is based on a combination of two-phase extractive EOC with subsequent pentafluoropropionylation and high temperature GC chromatography within an 8-min run. This method has advantages in the simple, rapid, sensitive, selective and reproducible quantification at concentrations ranging from pg/mL to high concentrations covering the serum estrogens levels in pregnant women. It can be a useful assay for a clinical diagnosis as well as a mining biomarker in estrogen-related disorders.

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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.2011.10.024.

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