A novel GC-MS method in urinary estrogen analysis from postmenopausal women with osteoporosis

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Abbreviations: GC-MS, gas chromatography-mass spectrometry; SPE, solid-phase extraction; SIM, selected-ion monitoring; LOD, limit of detection; LOQ, limit of quantification; TMS, trimethylsilylation; LC-MS, liquid chromatography-mass spectrometry.
Abstract

Estrogen metabolites play important roles in the development of female-related disorders and homeostasis of the bone. To improve detectability, a validated gas chromatography-mass spectrometry (GC-MS) method was conducted with a two-phase extractive ethoxycarbonylation (EOC) and subsequent perfluoroacylation with pentafluoropropionyl anhydride (PFPA) was introduced. The resulting samples were separated through a high temperature MXT-1 column within an 8-min run and were detected in the selected-ion monitoring mode. The optimized analytical conditions led to good separation with a symmetric peak shape for 19 estrogens as their EOC-PFP derivatives. The limit of quantification (LOQ) was 0.02 ~ 0.1 ng/mL for most estrogens analyzed except for 2-hydroxyestriol (0.5 ng/mL). The devised method was found to be linear (correlation coefficient, $r^2 > 0.995$) in the range from the LOQ to 40 ng/mL, whereas the precision (% CV) and accuracy (% bias) ranged from 1.4 to 10.5% and from 91.4 to 108.5%, respectively. The good sensitivity and selectivity of this method even allowed quantification of the estrogen metabolites in urine samples obtained from the postmenopausal female patients with osteoporosis. The present technique can be useful for clinical diagnosis as well as for a better understanding the pathogenesis of estrogen-related disorders in the low-level quantification.

Supplementary key words: estrogen profiling · ethoxycarbonylation · perfluoroacylation · postmenopausal women · osteoporosis · GC-MS
INTRODUCTION

Endogenous estrogens play an important role in the development of human cancers, such as breast, endometrial, ovary, thyroid and prostate cancer, as well as bone homeostasis (1–6). In general, higher levels of estrogens are associated with an increased risk of breast cancer and an estrogen deficiency can cause bone loss in postmenopausal women. Estrogens in women are biosynthesized in the ovaries and metabolized primarily through the NADPH-dependent hydroxylation (7).

Although the biological functions of the estrogen metabolism in the target tissues or cells are unclear, its metabolic changes are associated with a range of cancers and modulating bone density (1–6). 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. Hydroxylation on the A-ring occurs predominately at the C2 position and to a lesser extent, at the C4 position (8). The 2- and 4-hydroxy derivatives are further converted to 2-, 3-, and 4-methoxy estrogens by catechol-O-methyltransferase (9). 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 (10). According to the carcinogenic effect of breast cancer (1, 11, 12), catechol estrogens form quinones that react with DNA and form both stable and depurinating DNA adducts. Other metabolic pathways are also involved for the mitogenic and antiapoptotic effects of estrone, estradiol and some of their hydroxylated metabolites.

In contrast to many cancer studies (1–4), it is difficult to measure the concentrations of estrogen metabolites in postmenopausal women with osteoporosis to provide accurate quantification using conventional gas chromatography-mass spectrometry (GC-MS) based estrogen methods. These have been developed to measure the endogenous estrogen metabolites in biological specimens using radio- or enzyme immunoassay (RIA or EIA) (13, 14), and gas or liquid chromatography coupled to mass spectrometry (GC-MS (4, 15–18) or LC-MS (19–21). Although immunoassays have limited applicability due to an overestimation by cross-reactions (22–25), LC-MS-based profiling is a proven technique with high sensitivity and selectivity in estrogens analysis combined with chemical derivatization for the phenolic hydroxyl groups of estrogens as follows; (a) pentafluorobenzyl (PFB) derivatization with ECAPCI-MS (21),

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(b) dansyl (20) and picolinoyl (19) derivatizations with LC-ESI/MS, and (c) N-methyl-2-pyridyl (26), 1-(2,4-dinitro-5-fluorophenyl)-4,4,-dimethylpiperazine (27), or N-methyl-nicotinyl derivatizations with LC-ESI/MS (28). These are capable of quantifying estrogens in the low pg/mL ranges but the methods are time-consuming because they require derivatization (19) and a long analytical run (29). In high-resolution GC-MS analysis, estrogen analysis is also essential in blocking active hydrogens in both phenolic and aliphatic hydroxy groups. For this purpose, perfluoroacylation and trimethylsilylation are mainly performed (4, 15–18). As a different approach, alkoxycarbonylation, which is carried out with highly reacting agents for amino-, thiol-, imidazole- or phenolic hydroxy groups, can be suitable for direct reactions in aqueous media (17, 30–34). This direct-derivatization to make analytes extractable by non-polar organic solvents can be achieved simultaneously to provide rapid and reproducible results with excellent purification (17, 31, 32).

Here, we describe an optimized two-phase extractive ethoxycarbonylation (EOC) with ethylchloroformate (ECF) technique for a comprehensive analytical method of 19 endogenous estrogens including catechol estrogens to overcome their high polarity and instability in GC-MS based analysis. The subsequent perfluoroacylation with pentafluoropropionyl anhydride (PFPA) were combined. The resulting EOC-PFP estrogen metabolites were separated through a high temperature GC column, in which lower bleeding may achieve the results in better detectability with a short analytical run compared to a fused-silica GC column (35, 36). To demonstrate the utility of the present method in low-level detection, urine samples obtained from 100 postmenopausal female patients with osteoporosis were used. The chemical structures of the derivatives of estrogen metabolites new to the literature were identified and confirmed by mass spectral patterns.

**MATERIALS AND METHODS**

**Chemicals**

The 19 endogenous estrogens examined in this study (Table 1) were obtained from Steraloids (Newport, RI). The deuterium-labeled internal standard (IS), 2,4,16,16-\(d_4\)-estradiol (\(d_4\)-E2, isotopic purity \(\geq 98\%\)) was purchased from C/D/N isotopes (Pointe-Claire, Quebec, Canada). In solid-phase extraction (SPE), Oasis
HLB (3 cc, 60 mg; Waters, Milford, MA) 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 acquired from Sigma (St. Louis, MO). A solution of β-glucuronidase/arylsulfatase from *Helix pomatia* was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Anhydrous potassium carbonate anhydrous (K$_2$CO$_3$), triethylamine (TEA) and ethylchlorofomate (ECF) were obtained from J. T. Baker (Phillipsburg, NJ), Sigma, and Daejung Chemicals Co. (Shiheung, Gyengi, Korea), respectively. The acylation reagent, pentafluoropropionic anhydride (PFPA), and trimethylsilylating (TMS) agents, *N*-methyl-*N*-trifluorotrimethylsilyl acetamide (MSTFA), ammonium iodide (NH$_4$I), and dithioerythritol (DTE) were supplied by Sigma. All organic solvents used as the analytical and HPLC grades were purchased from Burdick & Jackson (Muskegan, MI). Deionized water was prepared using a Milli-Q purification system (Millipore, Billerica, MA).

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 ranging from 0.001 to 10 μg/mL. All standard solutions were stable for a minimum of 3 months at 4 °C in the teflon-sealed amber-glass vial.

**Calibration and quality control samples**

The urine samples used for calibration and quality-control (QC) purposes were prepared *in-house* as estrogen-free urine. The estrogen-free urine were prepared by percolating urine samples through Strata-X 30 μm polymeric reversed phase (3 mL, 60 mg; Phenomenex, UK). In this way, urinary estrogens are retained in the cartridges and then the eluates were collected. After checking the endogenous estrogens not presented by GC-MS, calibration samples were made up at 11 different concentrations ranging from 0.02 to 40 ng/mL. The quality control samples were also prepared at four different concentrations (0.1, 0.5, 2, and 10 ng/mL) with estrogen-free urine depending on the sensitivity and reference values of the urinary estrogens. All
samples contained L-ascorbic acid (1 mg/mL) to prevent the degradation of catechol estrogens during storage and sample preparation.

**Subjects and sample collection**

The first-morning urine samples were collected from 100 postmenopausal female patients with osteoporosis (age: 69.3 ± 5.9 years) at the Department of Endocrinology and Metabolism at the Severance Hospital (Seoul, Korea). All subjects underwent dual-energy X-ray absorptiometry (DEXA), which is a widely used technique to measure the bone mineral density (BMD) of the lumbar spine and upper part of the hip. The results are generally indicated by the T-score which is a comparison of the patient's BMD to that of a healthy thirty-year-old of the same gender and ethnicity. The criteria of the World Health Organization are as follows. Normal is a T-score of -1.0 or higher, osteopenia is defined as T < -1.0 and > -2.5, and osteoporosis is defined T ≤ -2.5. The levels of estrogens in the 100 postmenopausal female subjects with a T-score < -2.5 were corrected by the urinary creatinine values determined according to Jaffé method (37). All samples were stored at −20 °C until used.

**Optimization of ethoxycarbonylation**

To optimize the ethoxycarbonylation of a phenolic hydroxy group of estrogens, the experimental parameters affecting the derivatization efficiency were tested. To maximize the reaction yields, the effect of the catalyst was evaluated by adding TEA or pyridine in the range of 10 – 40 μL under pH 9 aqueous solutions. Under the addition of a catalyst, the pH of the reaction mixture was adjusted from 8 to 11 with a 5% K₂CO₃ solution, and also the volume of ECF was also tested in the range, 30 – 200 μL. In addition, various mechanical techniques including sonication, shaking, vortexing and incubation process were compared to increase the reactivity and reduce the reaction time. After optimizing the ethoxycarbonylation, the extraction solvent was evaluated to maximize the extraction efficiency with five different organic solvents, ethyl acetate (EA), t-butyl methyl ether (MTBE), ethyl acetate: n-hexane (2:3, v/v), n-pentane, and n-hexane.
After extractive ethoxycarbonylation, additional derivatization was processed to block the remaining aliphatic hydroxy or ketone groups of the estrogens prior to GC-MS analysis. Two different derivatization reagents were compared to obtain improved GC properties (Supplementary Fig. 1): (a) trimethylsilylation with 40 μL of MSTFA/NH₄I/DTE (500:4:2, v/w/w) for 20 min at 60 °C for both hydroxy and ketone groups, and (b) perfluoroacylation with 20 μL of PFPA in 100 μL of n-hexane at 50 °C for 30 min for only hydroxy groups. Two GC column systems were also evaluated using a fused-silica capillary column (Ultra-1; 25 m × 0.2 mm I.D., 0.33 μm film thickness; Agilent Technologies, Palo Alto, CA) and a stainless-steel capillary column (MXT-1; 30 m × 0.25 mm I.D., 0.25 μm film thickness; Restek Co., Bellefonte, PA).

**Urinary sample pretreatment**

The urine sample (2 mL) including 100 μL of 0.2% aqueous L-ascorbic acid was spiked to 15 μL of the internal standard, d₄-E2 (1 μg/mL). The samples were extracted with Oasis HLB™ SPE cartridges placed in a device fitted with a small peristaltic pump and operated at a low flow rate (< 1 mL/min) to improve the extraction efficiency during the SPE process. 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 then 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% K₂CO₃ solution, and 30 μL of TEA and 50 μL of ECF were added. After vortexing for 30 s, the sample was extracted twice with 2.5 mL non-polar solvent n-hexane. The organic solvent was evaporated in an N₂ evaporator at 40 °C and dried in a vacuum desiccator over P₂O₅-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 in an N₂ evaporator. Two microliters of the resulting product reconstituted with 40 μL of n-hexane was injected for GC-MS analysis in selected-ion monitoring (SIM) mode.

**Instrumental conditions**

GC-MS was performed with an Agilent 6890 Plus gas chromatograph interfaced with a single-
quadrupole Agilent 5975C MSD. The electron energy was 70 eV and the ion source temperature was 230 °C. Each sample (2 μL) was injected in split mode (10:1) 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 270 °C and 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 the carrier gas was set to 151.7 kPa. For quantitative analysis, the characteristic ions of each estrogen were determined as their EOC-PFP derivatives in SIM mode of the electron-impact ionization. To maximize the sensitivity, the characteristic ions were monitored in five different groups. Group 1, for the monitoring of 3-MeO-E2 and 3-MeO-E1, was set at $m/z$ 284 and 432 with a dwell time of 150 ms each and run from 2.60 to 3.95 min; group 2 (from 3.95 to 4.90 min) was set at $m/z$ 418, 422, and 580 with a dwell time of 100 ms for the analysis of 17α-E2, E3, $d_4$-17β-E2, 17β-E2, and 17-epi-E3; group 3 (from 4.90 to 5.50 min) was set at 270, 432, 448, and 580 for the monitoring of 16-epi-E3, 16α-OH-E1, 4-MeO-E2, and E1 with a dwell time of 75 ms each; group 4, for the monitoring of 2-MeO-E2, 16-keto-E2, 4-MeO-E1, and 2-MeO-E1, was set at $m/z$ 300, 432, and 448 with a dwell time of 100 ms each and run from 5.50 to 6.30 min; group 5 (from 5.50 to 8.00 min) was set at $m/z$ 286, 434, and 596 with a dwell time of 100 ms for the analysis of 2-OH-E3, 4-OH-E2, 2-OH-E2, 4-OH-E1, and 2-OH-E1. Peak identification was achieved by comparing the retention times and matching the height ratios of the characteristic ions (Table 1).

**Method validation**

The QC samples containing 19 estrogen metabolites were used over the course of 3 months and quantification was performed using the peak height ratios relative to that of IS. Least-squares regression analysis was performed on the peak height ratios against increasing amounts to obtain calibration linearity. The limit of detection (LOD) and quantification (LOQ) were defined to be the lowest concentration with a signal-to-noise (S/N) ratio > 3 for the LOD and 10 for the LOQ. The precision expressed as the coefficient of variation (% CV) and the accuracy as the percentage relative error (% bias) of the method were determined from the QC samples at three different concentrations (low; 0.1 or 0.5 ng/mL, medium; 2 ng/mL, and high;
10 ng/mL) based on the calibration range of each analyte. For the within-day repeatability, 5 replicates were analyzed, whereas the reproducibility was measured from the samples run over 5 different days. The extraction recovery was established using QC samples at three concentrations in triplicate for each estrogen by adding known amounts of the mixed working solutions to the estrogen-free urine samples. The absolute recovery was calculated by comparing the analytical results of the samples through overall sample preparation with those of standard samples without SPE and enzymatic hydrolysis that represent 100% recovery.

The stability of the analyte during sample collection and handling, which is a prerequisite of reliable quantification, was evaluated. The stability was measured by comparing the results of the samples analyzed before and after being exposed to the conditions for the stability assessment at three different concentrations in triplicate. First, the stability of the standard solutions was tested by standing at room temperature for 6 hr over the time required for sample preparation. Second, the freeze-thaw stability was determined after three freeze-thaw cycles. After storing three aliquots of QC samples at -20 °C for 24 hr, the samples were thawed at room temperature. When thawed completely, the samples were refrozen for 12 hr under the same conditions and these processes were repeated three times. Third, the short-term temperature stability was evaluated by thawing the QC samples at ambient temperature and then leaving them to stand at this temperature for 6 hr. Fourth, the post-preparative stability was evaluated by re-injecting the prepared samples after 6 hr (after one batch analysis of validation samples) and 30 hr (one day after being placed in the sample tray of auto-injector).

RESULTS

GC-MS Characteristics of the derivatives

To enhance both the specificity and sensitivity, a comprehensive derivatization for polar functional groups of estrogen analysis was carried out with the extractive EOC with ECF in the aqueous phase, which was applied successfully to protect the active hydrogens of the phenolic hydroxy group in estrogen
molecules as the direct-derivatization techniques (17). In the subsequent derivatization to block the remaining aliphatic hydroxy and ketone groups prior to GC analysis, TMS derivatization was initially tested and the results of the EOC-TMS derivatives were compared with the commonly used TMS derivatives (Supplementary Fig. 2). High-temperature GC techniques (36, 38, 39) used to separate high molecular weight and lipophilic compounds, which are not enough to elute from conventional fused-silica capillary GC columns, were examined for these EOC-TMS derivatives using a thermally-stable stainless-steel MXT-1 capillary column and were well separated in a 9 min-run (Supplementary Fig. 2). However, the EOC-TMS derivatives gave base peaks in low molecular mass ranges, which could be a cause of the decreasing sensitivity and selectivity in quantitative analysis. In the EOC-TMS derivatives of catechol estrogens, the precision (% CV) in the intra-assay (n=5) deviated by more than 20% at all QC concentrations, and the poor repeatability was not enhanced in both capillary column systems. In particular, 2-OH-E3 was not detectable with both TMS and EOC-TMS derivatization techniques in ng/mL urinary levels.

The aim of this study was to improve the detectability with good GC-EI/MS properties. Therefore, perfluoroacylation with PFPA for protection of the remaining aliphatic hydroxy groups was conducted as a subsequent derivatization. The chromatographic separation of 19 estrogens as their EOC-PFP derivatives was achieved with excellent peak shapes and higher responses within 8-min run (Fig. 1). To compare both the selectivity and sensitivity in three different derivatization methods, all estrogen metabolites were spiked into estrogen-free urine at the level of 1 ng/mL and analyzed. When the chromatographic properties were compared with the signal-to-noise (S/N) ratio, all EOC-PFP derivatives gave a better detectability with a 1.3 – 25 fold increase compared to the other two methods (Fig. 2). In addition, estradiol- or estriol-type compounds, which contain aliphatic hydroxy groups at the 16 and/or 17-position on the D-ring, showed a significantly higher S/N ratio, whereas the estrone-type compounds, which have no aliphatic hydroxy groups inducing subsequent derivatization with PFPA, were relatively low. The interpretation of the mass spectrometric patterns of estrogen metabolites also distinguished mono- and di-EOC-PFPA derivatives among the 19 estrogens (Table 1). The base peak of all mono-EOC-PFPA derivatives was the [M-72]^+ ion corresponding to the loss of a EtOCO-group, whereas the minor peaks were [M]^+ and [M-44; M-OEt]^+ ions.

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For five di-EOC-PFPA derivatives of catechol estrogens, the \([\text{M}-72-72; \text{M}-2\text{EtOCO}]^+\) ion formed the base peak, whereas \([\text{M}-72-44]^+, \ [\text{M}-72-44]^+, \ [\text{M}-44-44]^+, \ [\text{M}-72]^+, \ [\text{M}-44]^+\), and \([\text{M}]^+\) ions were observed as minor peaks. In addition, 3-MeO-E1 and 3-MeO-E2 without a phenolic hydroxy group generated the molecular ion as the base peak at \(m/z\) 284 and at \(m/z\) 432, respectively. These results may provide useful information on the chemical structures of the estrogens detected.

**Optimization of ethoxycarbonylation**

Although the dansyl derivatives of estrogens shows an improving detectability in LC-MS analysis (29), in this study, ethoxycarbonylation (30–34), 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 in GC-MS analysis. Initially, the contribution of the catalyst for complete ethoxycarbonylation was examined by comparing the chromatographic intensities of the EOC-derivatives in the scan range of \(m/z\) 100 – 650. In the absence of a catalyst, the methoxylated estrogens showed low reaction yields of 11 – 45% by a comparison with the addition of TEA (20 μL) that represent 100% efficacy. This may originate from the steric hindrance from the influence of a methoxy group adjacent to the phenolic hydroxy groups on an estrogen A-ring. To solve the problem, the addition of TEA and pyridine as a basic catalyst in the range, 10 – 40 μL in 1 mL of a buffer solution, was tested under the same reaction conditions (50 μL of ECF, pH 9 medium and shaking for 30 min). Although pyridine is commonly used as a suitable esterification catalyst (30, 33, 34), it was not selected in this study due to the difficulty in removing the excess reagent after derivatization. Increasing the TEA amount tended to maximize the reaction yields at 30 and 40 μL, and 30 μL of TEA was chosen because of its rapid evaporation rate.

In alkylxycarbonylation, an alkaline condition is essential to promote the deprotonation of the reactive species (30–32, 34) and the pH range 8 – 11 was tested (data not shown). Most estrogens showed good reaction yields (> 96%) in all ranges tested, whereas yields of five catechol estrogens were decreased significantly at pH > 9 even if 0.2% ascorbic acid was added to prevent oxidation of the catechol moieties. The optimal pH was adjusted to 8 – 8.5 using a 5% \(\text{K}_2\text{CO}_3\) solution. To set an optimal volume of ECF, the

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amount of ECF was also tested with volumes of 30 – 200 μL in 1 mL of aqueous media at pH 8. The highest reaction yield was obtained in the range of 50 – 200 μL ECF. Hence, 50 μL of ECF was used in this study. Further experiments were carried out to optimize the reaction time. The experiment involved increasing the time (10 s – 30 min) combined with various mixing techniques (sonication, mechanical shaking and vortexing) and reaction temperatures (25 and 50 °C). Both mechanical shaking and sonication at 50 °C improved the reaction yields and increased the reaction time, but the increase was not significant compared to vortexing at 25 °C in a few seconds. Therefore, vortexing for 30 seconds was chosen as the simplest condition.

The sample pH did not affect on extraction efficiency, and extraction was performed without additional pH adjustment after ethoxycarbonylation. As the extraction solvents, five different organic solvents, ethyl acetate (EA), t-butyl methyl ether (MTBE), a mixture of EA and n-hexane (2:3, v/v), n-pentane, and n-hexane were tested to assess the enhanced extraction efficiency and reduced matrix interference. The order of diminishing matrix noise was n-hexane ≈ n-pentane > EA : n-hexane (2:3) > MTBE > EA. When relatively polar organic solvents, EA, MTBE and EA : n-hexane (2:3), were used, the chromatographic interference derived from the urinary backgrounds was increased, which is responsible for the poor selectivity and sensitivity. In contrast, extraction with nonpolar solvents, such as n-hexane and n-pentane, considering the increased lipophilicity of estrogens after ethoxycarbonylation was quite effective in removing disturbing polar substances. The n-hexane was used as an optimal extraction solvent.

METHOD VALIDATION

Method validation requires evaluations of the accuracy, precision, selectivity, sensitivity, reproducibility and stability using spiked samples prepared with the estrogen-free urine. The LOD and LOQ, which are sample concentrations required to give a higher signal-to-noise (S/N) ratio > 3 and 10, respectively, were also evaluated (Table 2). The LOQs of the EOC-PFP derivatives with the estrone-type moiety were 0.06 ~ 0.1 ng/mL, whereas those of most estrogens with estradiol- or estriol-type moieties ranged from 0.02 to 0.04 ng/mL, except for 2-OH-E3 (0.5 ng/mL). This may due to the relatively low recovery obtained from n-
hexane extraction after ethoxycarbonylation. The overall recoveries of the EOC-PFP derivatives ranged from 75.3 to 109.5%, except 48.7% for 2-OH-E3.

The calibration curve consisted of a blank sample (matrix sample processed without an internal standard), a zero sample (matrix sample processed with an internal standard), and eleven samples from LOQ to the expected range in the sample. The devised method was linear over a 10³-fold concentration range with the correlation coefficient ($r^2 > 0.995$) for all estrogens analyzed. The precision and accuracy were determined by analyzing the QC samples acquired for the intra- and interday assays at 3 different levels of low (0.1 or 0.5 ng/mL), medium (2 ng/mL), and high (10 ng/mL) concentrations. The intra-day ($n = 5$) precision (expressed as % CV) ranged from 1.6 to 11.5%, whereas accuracies (expressed as % bias) ranged from 94.0 to 109.5%, and inter-day ($n = 5$) precision (% CV) and accuracy (% bias) ranged from 1.4 to 10.5% and from 91.4 to 108.5%, 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 the analytical process as described in the Experimental section (Supplementary Table 1). Both stock solutions and QC samples were prepared freshly and L-ascorbic acid (1 mg/mL) was added to prevent oxidative degradation of the catechol estrogens. The standard solutions were stable at -20 °C for three months and at room temperature for 6 hr. The short-term stability, which was tested by thawing the QC samples at 25 °C and leaving them to stand for 6 hr, showed no significant changes (< ± 15% RSD) in concentration under the conditions tested. The freeze/thaw stability was evaluated from the concentration of aliquot not subjected to freeze/thaw cycles as a reference. The stability of the estrogens was also demonstrated in urine samples subjected to three freeze/thaw cycles. The overall differences between the cycles were not significant within a 15% deviation in all estrogens. Repeated the freezing and thawing cycles did not appear to affect the concentration of estrogens. Instability can only occur in the sample matrix but also in the prepared samples. Hence, it is important to test the post-preparative stability under the conditions of analysis including autosampler conditions for the expected maximum time of an analytical run 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 one day after being placed in the sample-tray ($\pm 12\% \text{ RSD}$).

**Application into the postmenopausal women with osteoporosis**

The usefulness of this novel method for quantifying low-levels of endogenous estrogens was demonstrated with urine samples obtained from 100 postmenopausal female patients with osteoporosis (Fig. 3). Among the 19 estrogens monitored, E1, 17β-E2, E3, 17-epi-E3, 16-epi-E3, 16α-OH-E1, 16-keto-E2, and 2-MeO-E2 were detected in all 100 urine samples, whereas 2-MeO-E1, 3-MeO-E1, 2-OH-E1, 2-OH-E2, 4-OH-E1, and 4-OH-E2 detected approximately 50% of the total samples analyzed. Among the 8 estrogens with a total of above 90% incidence, E1 was the most abundant estrogen, followed by E3 and 2-MeO-E1.

**DISCUSSION**

Estrogen metabolites have one or two phenolic hydroxy groups and other polar groups, such as aliphatic hydroxy or ketone (7, 8). When high-resolution capillary GC-MS is used for estrogen analysis (15–18), it is essential to block the active hydrogens in these polar functional groups. Although TMS derivatives are commonly used in GC-MS-based estrogen analysis (15, 16), it is not enough to reach the sensitivity for quantification of estrogen metabolites in urine samples at sub-ng/mL levels, and performed with a time consuming process. As a different approach, the direct-derivatization in aqueous solutions to make them extractable by non-polar organic solvents was more preferred. With extractive two-phase AOC procedures, derivatization and extraction of analytes in aqueous solutions are achieved in the nearly same time (17, 31, 32). Accordingly, it was desirable to improve the volatility and GC properties of lipophilic molecules. This might be accomplished by performing AOC with less bulky methyl-, or ethyl chloroformate, with subsequent perfluoroacylation or alkylsilylation of the remaining active hydrogen atoms. However, attempts were rarely made to adopt this method for the biomolecule analysis.

In our previous study, a high temperature stainless-steel capillary column was successfully utilized for
the analysis of lipid molecules as their TMS derivatives with good chromatographic resolution (36, 39), while fused-silica capillary columns have poor chromatographic properties or long retention times. Initial studies with a fused-silica capillary column above 300 °C for a while produced poor results because fused-silica capillary column become brittle over time above 300 °C and all analytes were eluted as broad peaks accompanied by extraneous peaks. In continuation of steroid metabolic studies, the present study was undertaken to determine optimal HTGC-MS conditions for analyzing 19 estrogen metabolites as their EOC-PFP derivatives using a thermally stable stainless-steel capillary column. Lower bleeding achieved by enhanced thermo-stability results in better detectability.

Using the EOC-PFP derivatization, 16α-OH-E1 was separated completely from 16-keto-E2, whereas they were co-eluted in both TMS and EOC-TMS experiments (15). Both compounds have a similar molecular weight and mass fragments. In general, 16α-OH-E1 stimulated cell proliferation in breast cancer cell lines and showed estrogenic and genotoxic potential in oxidative stress-induced biological actions (40). Accurate quantification is very important and EOC-PFP derivatization gave selective and sensitive detection in urine samples. Although three estrogens (e.g., 16-epi-E3, 16α-OH-E1, and 4-MeO-E2) were co-eluted in this method, they were differentiated by SIM analysis because of their different characteristic ions at m/z 580, 432, and 448, respectively (Table 1).

Dansyl chloride is commonly used for improving the detectability of estrogens with phenolic hydroxyl groups in LC-MS analysis (29). The methods are capable of quantitatively measuring 15 estrogen metabolites and have been applied successfully to urine samples from premenopausal and postmenopausal healthy women. However, there are limitations including a long analytical time (70 min) for the separation of 15 estrogens, which might not be effective in large-scale applications. In this study, ethoxycarbonylation (30–34), 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 in GC-MS analysis.

Owing to these low concentrations in postmenopausal women, many studies have generally focused on measuring the abundant estrogens, such as E1, 17β-E2, and E3. Recently, a LC-MS/MS method provided
more information on 15 estrogen metabolites present in urine samples obtained from healthy premenopausal and postmenopausal women. Five (E1, 17β-E2, E3, 2-OH-E1, 16-keto-E2) of these 15 estrogens were calculated as the abundant estrogens in both two groups (20, 25). In addition, few studies have evaluated the excretion of urinary estrogen metabolites in postmenopausal osteoporosis, which is known to be due primarily to an estrogen deficiency (6). To examine the estrogen metabolisms in postmenopausal osteoporosis, the urinary levels of hydroxylated and methoxylated estrogens were measured using immunoassay kits and GC-MS. On the other hand, the analytical sensitivities in an accurate measurement of low-level estrogen metabolites are insufficient. The present technique would make it possible to have better sensitivity and selectivity as a comprehensive GC-MS based method.

In conclusion, a comprehensive GC-MS method was developed as a practical assay to quantify 19 urinary estrogens. The devised technique was based on a combination of two-phase extractive EOC with the subsequent pentafluoropropionylation and GC separation with a high temperature GC column within 8-min run. The method has the advantages of simple, rapid, sensitive, selective and reproducible quantification at pg/mL levels of urinary estrogen metabolites. The good sensitivity of this method even allowed measurements of the estrogen metabolites in the urine of post-menopausal female patients with osteoporosis. This can be a useful technique for a clinical diagnosis and as a mining biomarker in estrogen-related disorders.

ACKNOWLEDGEMENT

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REFERENCES


[20]
FIGURE LEGENDS

**Fig. 1.** Total-ion chromatogram in GC-SIM/MS analysis of the 19 estrogens as their EOC-PFP derivatives. The 19 estrogens (10 ng/mL each) were spiked into the steroid-free urine 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. The characteristic ions were monitored in five different groups. Group 1, for the monitoring of 1 and 2, was set at \( m/z \) 284 and 432 and run from 2.60 to 3.95 min; group 2 (from 3.95 to 4.90 min) was set at \( m/z \) 418, 422, and 580 for the analysis of 3 to 6; group 3 (from 4.90 to 5.50 min) was set at 270, 432, 448, and 580 for the monitoring of 7 to 10; group 4, for the monitoring of 11 to 14, was set at \( m/z \) 300, 432, and 448 and run from 5.50 to 6.30 min; group 5 (from 5.50 to 8.00 min) was set at \( m/z \) 286, 434, and 596 for the analysis of 15 to 19.

**Fig. 2.** Comparative detectability of the three different derivatization techniques. Nineteen estrogen metabolites were spiked at 1 ng/mL urinary concentration and analyzed. As representative extracted-ion chromatograms based on the quantitative ions of individual compounds, catechol estrone and estradiol are plotted without smoothing.

**Fig. 3.** Urinary estrogen levels obtained from 100 postmenopausal female patients with osteoporosis. All quantitative data was corrected by the urinary creatinine values.
Table 1. The GC-MS information for quantitative analysis of 19 estrogens as their EOC-PFP derivatives

<table>
<thead>
<tr>
<th>Compounds (Trivial name)</th>
<th>Abbreviation</th>
<th>Molecular ion</th>
<th>Characteristic ion&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Quantitative ion&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-methoxy-17β-estradiol</td>
<td>3-MeO-E2</td>
<td>432</td>
<td>432, 404</td>
<td>432 [M]+</td>
<td>3.26</td>
</tr>
<tr>
<td>3-methoxyestrone</td>
<td>3-MeO-E1</td>
<td>284</td>
<td>284, 227</td>
<td>284 [M]+</td>
<td>3.61</td>
</tr>
<tr>
<td>17α-estradiol</td>
<td>17α-E2</td>
<td>490</td>
<td>490, 446, 418</td>
<td>418 [M-72]+</td>
<td>4.35</td>
</tr>
<tr>
<td>Estriol</td>
<td>E3</td>
<td>652</td>
<td>652, 608, 580</td>
<td>580 [M-72]+</td>
<td>4.41</td>
</tr>
<tr>
<td>17β-estradiol</td>
<td>17β-E2</td>
<td>490</td>
<td>490, 446, 418</td>
<td>418 [M-72]+</td>
<td>4.67</td>
</tr>
<tr>
<td>17-epiestriol</td>
<td>17-epi-E3</td>
<td>652</td>
<td>652, 608, 580</td>
<td>580 [M-72]+</td>
<td>4.76</td>
</tr>
<tr>
<td>16-epiestriol</td>
<td>16-epi-E3</td>
<td>652</td>
<td>652, 608, 580</td>
<td>580 [M-72]+</td>
<td>5.19</td>
</tr>
<tr>
<td>16α-hydroxyestrone</td>
<td>16α-OH-E1</td>
<td>504</td>
<td>504, 460, 432</td>
<td>432 [M-72]+</td>
<td>5.19</td>
</tr>
<tr>
<td>4-methoxy-17β-estradiol</td>
<td>4-MeO-E2</td>
<td>520</td>
<td>520, 476, 448</td>
<td>448 [M-72]+</td>
<td>5.22</td>
</tr>
<tr>
<td>Estrone</td>
<td>E1</td>
<td>342</td>
<td>342, 298, 270</td>
<td>270 [M-72]+</td>
<td>5.30</td>
</tr>
<tr>
<td>2-methoxy-17β-estradiol</td>
<td>2-MeO-E2</td>
<td>520</td>
<td>520, 476, 448</td>
<td>448 [M-72]+</td>
<td>5.50</td>
</tr>
<tr>
<td>16-keto-17β-estradiol</td>
<td>16-keto-E2</td>
<td>504</td>
<td>504, 460, 432</td>
<td>432 [M-72]+</td>
<td>5.59</td>
</tr>
<tr>
<td>4-methoxyestrone</td>
<td>4-MeO-E1</td>
<td>372</td>
<td>372, 328, 300</td>
<td>300 [M-72]+</td>
<td>5.86</td>
</tr>
<tr>
<td>2-methoxyestrone</td>
<td>2-MeO-E1</td>
<td>372</td>
<td>372, 328, 300</td>
<td>300 [M-72]+</td>
<td>6.10</td>
</tr>
<tr>
<td>4-hydroxyestrone</td>
<td>4-OH-E1</td>
<td>430</td>
<td>430, 386, 358, 342, 314, 286</td>
<td>286 [M-72-72]+</td>
<td>7.26</td>
</tr>
<tr>
<td>2-hydroxyestrone</td>
<td>2-OH-E1</td>
<td>430</td>
<td>430, 386, 358, 342, 314, 286</td>
<td>286 [M-72-72]+</td>
<td>7.50</td>
</tr>
</tbody>
</table>

<sup>a</sup>All estrogens were derivatized with the acylation agent, pentafluoropropionnic anhydride (PFPA) for 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 phenolic and/or aliphatic hydroxy groups. All ions are given within 30% of the base peak.

<sup>b</sup>Base peaks were chosen as the quantitative ions of estrogens.
Table 2. The validation results of the overall method in the intra- and inter-day assays

<table>
<thead>
<tr>
<th>Compounds (Abbreviation)</th>
<th>LOD$^a$ (ng/mL)</th>
<th>LOQ$^b$ (ng/mL)</th>
<th>Recovery$^c$ (%)</th>
<th>Linearity$^d$ ($r^2$)</th>
<th>Intra-day ($n=5$)</th>
<th>Inter-day ($n=5$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CV$^e$ (%)</td>
<td>Accuracy$^e$ (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CV (%)</td>
<td>Accuracy (%)</td>
</tr>
<tr>
<td>E1</td>
<td>0.04</td>
<td>0.08</td>
<td>95.2</td>
<td>0.9984</td>
<td>4.8</td>
<td>109.5</td>
</tr>
<tr>
<td>17β-E2</td>
<td>0.01</td>
<td>0.02</td>
<td>103.9</td>
<td>0.9997</td>
<td>1.6</td>
<td>109.2</td>
</tr>
<tr>
<td>E3</td>
<td>0.01</td>
<td>0.04</td>
<td>75.3</td>
<td>0.9974</td>
<td>4.7</td>
<td>106.3</td>
</tr>
<tr>
<td>2-OH-E1</td>
<td>0.05</td>
<td>0.10</td>
<td>90.8</td>
<td>0.9953</td>
<td>11.5</td>
<td>101.5</td>
</tr>
<tr>
<td>2-OH-E2</td>
<td>0.01</td>
<td>0.02</td>
<td>96.7</td>
<td>0.9978</td>
<td>4.0</td>
<td>99.7</td>
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<tr>
<td>4-OH-E1</td>
<td>0.05</td>
<td>0.10</td>
<td>80.7</td>
<td>0.9972</td>
<td>11.6</td>
<td>97.7</td>
</tr>
<tr>
<td>4-OH-E2</td>
<td>0.01</td>
<td>0.04</td>
<td>93.3</td>
<td>0.9985</td>
<td>6.8</td>
<td>97.9</td>
</tr>
<tr>
<td>2-MeO-E1</td>
<td>0.02</td>
<td>0.04</td>
<td>92.9</td>
<td>0.9980</td>
<td>7.9</td>
<td>105.4</td>
</tr>
<tr>
<td>2-MeO-E2</td>
<td>0.01</td>
<td>0.02</td>
<td>103.8</td>
<td>0.9997</td>
<td>4.1</td>
<td>98.7</td>
</tr>
<tr>
<td>3-MeO-E1</td>
<td>0.05</td>
<td>0.10</td>
<td>86.6</td>
<td>0.9977</td>
<td>10.9</td>
<td>100.0</td>
</tr>
<tr>
<td>3-MeO-E2</td>
<td>0.01</td>
<td>0.02</td>
<td>95.0</td>
<td>0.9984</td>
<td>4.6</td>
<td>100.2</td>
</tr>
<tr>
<td>4-MeO-E1</td>
<td>0.04</td>
<td>0.10</td>
<td>98.7</td>
<td>0.9976</td>
<td>5.6</td>
<td>107.9</td>
</tr>
<tr>
<td>4-MeO-E2</td>
<td>0.01</td>
<td>0.02</td>
<td>102.3</td>
<td>0.9996</td>
<td>2.3</td>
<td>106.8</td>
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<tr>
<td>17-epi-E3</td>
<td>0.01</td>
<td>0.04</td>
<td>107.0</td>
<td>0.9994</td>
<td>7.1</td>
<td>101.3</td>
</tr>
<tr>
<td>16-epi-E3</td>
<td>0.01</td>
<td>0.04</td>
<td>109.4</td>
<td>0.9993</td>
<td>6.1</td>
<td>101.5</td>
</tr>
<tr>
<td>17α-E2</td>
<td>0.02</td>
<td>0.04</td>
<td>109.5</td>
<td>0.9994</td>
<td>3.4</td>
<td>96.3</td>
</tr>
<tr>
<td>16α-OH-E1</td>
<td>0.02</td>
<td>0.06</td>
<td>100.1</td>
<td>0.9986</td>
<td>9.2</td>
<td>108.9</td>
</tr>
<tr>
<td>16-keto-E2</td>
<td>0.02</td>
<td>0.06</td>
<td>102.7</td>
<td>0.9981</td>
<td>6.9</td>
<td>94.0</td>
</tr>
<tr>
<td>2-OH-E3</td>
<td>0.10</td>
<td>0.50</td>
<td>48.7</td>
<td>0.9950</td>
<td>24.8</td>
<td>102.2</td>
</tr>
</tbody>
</table>

$^a$The limit of detection was measured at S/N ratio > 3. $^b$Limit of quantification was measured at S/N ratio > 10. $^c$Absolute recoveries were calculated by comparing the peak height ratios of the samples using the described method with those of their non-extracted counterparts from three different QC concentrations in 5 replicates. $^d$Calibration ranges were from the LOQ level to 40 ng/mL for each analyte and the linearity was measured as the mean levels of data through 5 different days. $^e$Precision and accuracy were expressed as the mean values of data obtained from three QC samples through intra- and inter-day assays.
Fig. 1.
Fig. 2.

2-OH-E1

TMS

m/z 502

S/N=41.1

m/z 502

S/N=41.1

EOC-TMS

m/z 343

S/N=12.3

m/z 343

S/N=12.3

EOC-PFPA

m/z 286

S/N=66.5

m/z 286

S/N=66.5

2-OH-E2

TMS

m/z 504

S/N=35.9

m/z 504

S/N=35.9

EOC-TMS

m/z 360

S/N=40.5

m/z 360

S/N=40.5

EOC-PFPA

m/z 434

S/N=384.2

m/z 434

S/N=384.2
Fig. 3.

( ): detection incidence (%)

ng/g of creatinine

$E_1$ 17p-E2 $E_3$ 2-OHE1 2-OHE2 4-OHE1 4-OHE2 2-MeO-E1 2-MeO-E2 3-MeO-E1 17-epi-E3 16-epi-E3 16α-OHE1 16α-Keto-E2