

# A novel GC-MS method in urinary estrogen analysis from postmenopausal women with osteoporosis<sup>§</sup>

Ju-Yeon Moon,<sup>\*,†</sup> Kwang Joon Kim,<sup>§</sup> Myeong Hee Moon,<sup>†</sup> Bong Chul Chung,<sup>\*</sup> and Man Ho Choi<sup>1,\*</sup>

Future Convergence Research Division,<sup>\*</sup> Korea Institute of Science and Technology, Seoul, Korea; and Department of Chemistry,<sup>†</sup> College of Science, and Department of Internal Medicine,<sup>§</sup> College of Medicine, Yonsei University, Seoul, Korea

**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 two-phase extractive ethoxycarbonylation (EOC) and subsequent pentafluoropropionyl (PFP) derivatization 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 (SIM) 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 from 0.02 to ~0.1 ng/ml for most estrogens analyzed, except for 2-hydroxyestriol (0.5 ng/ml). The devised method was found to be linear ( $r^2 > 0.995$ ) 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 to better understand the pathogenesis of estrogen-related disorders in low-level quantification.—Moon, J.-Y., K. J. Kim, M. H. Moon, B. C. Chung, and M. H. Choi. A novel GC-MS method in urinary estrogen analysis from postmenopausal women with osteoporosis. *J. Lipid Res.* 2011. 52: 1595–1603.

**Supplementary key words** steroid profiling • ethoxycarbonylation • hormones

Endogenous estrogens play an important role in the development of human cancers, such as breast, endometrial, ovary, thyroid, and prostate cancer, as well as in bone homeostasis (1–6). In general, higher levels of estrogens are associated with an increased risk of breast cancer, but 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 estrogen metabolism in the target tissues or cells are unclear, its metabolic changes are associated with a range of cancers and in 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 16 $\alpha$  position of the D-ring produces 16 $\alpha$ -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

Abbreviations: BMD, bone mineral density; DEXA, dual-energy X-ray absorptiometry; DTE, dithioerythritol; EA, ethyl acetate; ECF, ethylchloroformate; EIA, enzyme immunoassay; EOC, ethoxycarbonylation; IS, internal standard; LOD, limit of detection; LOQ, limit of quantification; MSTFA, *N*-methyl-*N*-trifluoromethylsilyl acetamide; MTBE, *t*-butyl methyl ether; PFP, pentafluoropropionyl; QC, quality control; SIM, selected ion monitoring; S/N ratio, signal-to-noise ratio; RSD, relative standard deviation; SPE, solid-phase extraction; TEA, triethylamine.

<sup>1</sup>To whom correspondence should be addressed.

e-mail: mh\_choi@kist.re.kr

<sup>§</sup>The online version of this article (available at <http://www.jlr.org>) contains supplementary data in the form of two figures and one table.

This study was supported by Grants from the Korean Institute of Science and Technology (KIST) and the Converging Research Center Program through the Ministry of Education, Science, and Technology (2010K001117).

Manuscript received 1 April 2011 and in revised form 16 May 2011.

Published, JLR Papers in Press, May 21, 2011  
DOI 10.1194/jlr.D016113

spectrometry (GC-MS)-based estrogen methods. These have been developed to measure the endogenous estrogen metabolites in biological specimens using RIA or enzyme immunoassay (EIA) (13, 14), and 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 estrogen analysis combined with chemical derivatization for the phenolic hydroxyl groups of estrogens as follows: *a*) pentafluorobenzyl (PFB) derivatization with ECAPCI-MS (21), *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 methods are capable of quantifying estrogens in the low pg/ml ranges, but they are time consuming because they require derivatization (19) and a long analytical run (29). In high-resolution GC-MS analysis, estrogen analysis is 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 nonpolar 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. Subsequent pentafluoropropionyl (PFP)

derivatization was combined. The resulting EOC-PFP estrogen metabolites were separated through a high-temperature GC column, in which lower bleeding achieved results in better detectability with a short analytical run compared with 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*<sub>4</sub>-17β-estradiol (*d*<sub>4</sub>-E2, isotopic purity ≥ 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 de-ionized 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/aryl-sulfatase from *Helix pomatia* was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Anhydrous potassium carbonate anhydrous (K<sub>2</sub>CO<sub>3</sub>), triethylamine (TEA), and ethylchloroformate (ECF) were obtained from J. T. Baker (Phillipsburg, NJ), Sigma, and Daejung Chemical Co. (Shiheung, Gyengi, Korea), respectively. The perfluoroacylation reagent, pentafluoropropionic anhydride, and trimethylsilylating (TMS) agents, *N*-methyl-*N*-trifluorotrimethylsilyl acetamide (MSTFA), ammonium iodide (NH<sub>4</sub>I), and dithioerythritol (DTE), were supplied by Sigma. All organic solvents used as the analytical and HPLC grades were purchased from Burdick and Jackson (Muskegan, MI). Deionized water was prepared using a Milli-Q purification system (Millipore, Billerica, MA).

TABLE 1. GC-MS information for quantitative analysis of 19 estrogens as their EOC-PFP derivatives

Compounds(Trivial Name)	Abbreviation	Molecular Ion	Characteristic Ion <sup>a</sup>	Quantitative Ion <sup>b</sup>	Retention Time(min)
3-methoxy-17β-estradiol	3-MeO-E2	432	432, 404	432 [M] <sup>+</sup>	3.26
3-methoxyestrone	3-MeO-E1	284	284, 227	284 [M] <sup>+</sup>	3.61
17α-estradiol	17α-E2	490	490, 446, 418	418 [M-72] <sup>+</sup>	4.35
Estriol	E3	652	652, 608, 580	580 [M-72] <sup>+</sup>	4.41
17β-estradiol	17β-E2	490	490, 446, 418	418 [M-72] <sup>+</sup>	4.67
17-epiestriol	17-epi-E3	652	652, 608, 580	580 [M-72] <sup>+</sup>	4.76
16-epiestriol	16-epi-E3	652	652, 608, 580	580 [M-72] <sup>+</sup>	5.19
16α-hydroxyestrone	16α-OH-E1	504	504, 460, 432	432 [M-72] <sup>+</sup>	5.19
4-methoxy-17β-estradiol	4-MeO-E2	520	520, 476, 448	448 [M-72] <sup>+</sup>	5.22
Estrone	E1	342	342, 298, 270	270 [M-72] <sup>+</sup>	5.30
2-methoxy-17β-estradiol	2-MeO-E2	520	520, 476, 448	448 [M-72] <sup>+</sup>	5.50
16-keto-17β-estradiol	16-keto-E2	504	504, 460, 432	432 [M-72] <sup>+</sup>	5.59
4-methoxyestrone	4-MeO-E1	372	372, 328, 300	300 [M-72] <sup>+</sup>	5.86
2-methoxyestrone	2-MeO-E1	372	372, 328, 300	300 [M-72] <sup>+</sup>	6.10
2-hydroxyestriol	2-OH-E3	740	740, 696, 652, 624, 596	596 [M-72-72] <sup>+</sup>	6.52
4-hydroxy-17β-estradiol	4-OH-E2	578	578, 534, 506, 490, 462, 434	434 [M-72-72] <sup>+</sup>	6.63
2-hydroxy-17β-estradiol	2-OH-E2	578	578, 534, 506, 490, 462, 434	434 [M-72-72] <sup>+</sup>	6.88
4-hydroxyestrone	4-OH-E1	430	430, 386, 358, 342, 314, 286	286 [M-72-72] <sup>+</sup>	7.26
2-hydroxyestrone	2-OH-E1	430	430, 386, 358, 342, 314, 286	286 [M-72-72] <sup>+</sup>	7.50

<sup>a</sup>All estrogens were derivatized with the perfluoroacylation agent pentafluoropropionic anhydride for hydroxy groups after ethoxycarbonylation (EOC) with ethyl chloroformate 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.

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 methanol-ascorbic acid solution at various concentrations ranging from 0.001 to 10  $\mu$ g/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 by percolating urine samples through a Strata-X (3 ml, 60 mg; Phenomenex, UK). Urinary estrogens were retained in the cartridges, and the eluates were collected. After checking the endogenous estrogens not presented by GC-MS, calibration samples were made up of 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  $\pm$  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 compares 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  $T \leq -2.5$ . 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  $-20^\circ\text{C}$  until used.

### Optimization of ethoxycarbonylation

To optimize the ethoxycarbonylation of a phenolic hydroxy group of estrogens, the experimental parameters affecting 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  $\mu$ l 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%  $\text{K}_2\text{CO}_3$  solution. The volume of ECF was tested in the range of 30–200  $\mu$ l. In addition, various mechanical techniques, including sonication, shaking, vortexing, and incubation, were compared to increase the reactivity and reduce 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 carried out to block the remaining aliphatic hydroxy or ketone groups of estrogens prior to GC-MS analysis. Two different derivatization reagents were compared to obtain improved GC properties (supplementary Fig. 1): *a*) trimethylsilylation with 40  $\mu$ l

of MSTFA/ $\text{NH}_4\text{I}$ /DTE (500:4:2, v/w/w) for 20 min at  $60^\circ\text{C}$  for both hydroxy and ketone groups and *b*) perfluoroacylation with 20  $\mu$ l of pentafluoropropionic anhydride in 100  $\mu$ l of *n*-hexane at  $50^\circ\text{C}$  for 30 min only for hydroxy groups. Two GC column systems were also evaluated using a fused-silica capillary column (Ultra-1; 25 m  $\times$  0.2 mm I.D., 0.33  $\mu$ m film thickness; Agilent Technologies, Palo Alto, CA) and a stainless steel capillary column (MXT-1; 30 m  $\times$  0.25 mm I.D., 0.25  $\mu$ m film thickness; Restek Co., Bellefonte, PA).

### Urinary sample pretreatment

The urine sample (2 ml) including 100  $\mu$ l of 0.2% aqueous L-ascorbic acid was spiked to 15  $\mu$ l of the internal standard,  $d_4$ -E2 (1  $\mu$ g/ml). The samples were extracted with Oasis HLB<sup>TM</sup> 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 each sample on a cartridge, it 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  $\mu$ l of aqueous 0.2% L-ascorbic acid, and 50  $\mu$ l of  $\beta$ -glucuronidase/arylsulfatase. After incubation at  $55^\circ\text{C}$  for 3 h, the solution was adjusted to pH 8 with a 5%  $\text{K}_2\text{CO}_3$  solution, and then 30  $\mu$ l of TEA and 50  $\mu$ l of ECF were added. After vortexing for 30 s, the sample was extracted twice with 2.5 ml nonpolar solvent *n*-hexane. The organic solvent was evaporated in an  $\text{N}_2$  evaporator at  $40^\circ\text{C}$  and dried in a vacuum desiccator over  $\text{P}_2\text{O}_5$ -KOH for at least 30 min. Finally, the dried residue was derivatized with 20  $\mu$ l of pentafluoropropionic anhydride in 100  $\mu$ l of *n*-hexane at  $50^\circ\text{C}$  for 30 min, and then evaporated in an  $\text{N}_2$  evaporator. Two microliters of the resulting product reconstituted with 40  $\mu$ l 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  $230^\circ\text{C}$ . Each sample (2  $\mu$ l) was injected in split mode (10:1) at  $280^\circ\text{C}$  and separated through a MXT-1 (30 m  $\times$  0.25 mm I.D., 0.25  $\mu$ m film thickness, Silcosteel-treated stainless steel) cross-linked dimethyl polysiloxane capillary column. The oven temperature was initially  $270^\circ\text{C}$ , increased to  $300^\circ\text{C}$  at  $6^\circ\text{C}/\text{min}$ , and finally increased to  $330^\circ\text{C}$  using a  $10^\circ\text{C}/\text{min}$  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  $17\alpha$ -E2, E3,  $d_4$ - $17\beta$ -E2,  $17\beta$ -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,  $16\alpha$ -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; and 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 three 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 intraday repeatability, five replicates were analyzed, whereas the reproducibility was measured from the samples run over five 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 represented 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 h 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  $-20^{\circ}\text{C}$  for 24 h, the samples were thawed at room temperature. When thawed completely, the samples were refrozen for 12 h under the same conditions. This process was 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 h. Fourth, the postpreparative stability was evaluated by re-injecting the prepared samples after 6 h (after one batch analysis of validation samples) and 30 h (one day after being placed in the sample tray of the auto-injector).

## RESULTS

### GC-MS characteristics of the derivatives

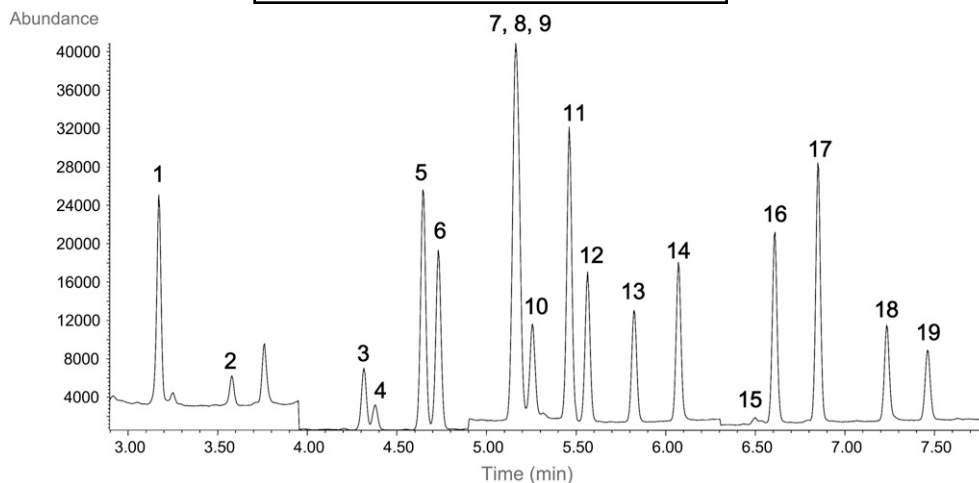
To enhance both specificity and sensitivity, a comprehensive derivatization for polar functional groups of estrogen analysis was carried out with the extractive EOC with ECF in the aqueous phase, which was applied successfully to protect the active hydrogens of the phenolic hydroxy group in estrogen molecules as the direct-derivatization technique (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. II). High-temperature GC techniques (36, 38, 39), which can separate high molecular weight or lipophilic compounds that cannot be eluted using conventional fused-silica capillary GC columns, were examined. By using a thermally stable, stainless steel MXT-1 capillary col-

umn, the EOC-TMS derivatives were well separated in a 9 min run (supplementary Fig. II). However, the EOC-TMS derivatives gave base peaks in low molecular mass ranges, which could cause 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, PFP derivatization 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 runs (Fig. 1). To compare both 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 S/N ratio, all EOC-PFP derivatives gave better detectability with a 1.3- to 25-fold increase compared with 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 pentafluoropropionic anhydride, were relatively low. The interpretation of the mass spectrometric patterns of estrogen metabolites also distinguished mono- and di-EOC-PFP derivatives among the 19 estrogens (Table 1). The base peak of all mono-EOC-PFP derivatives was the  $[\text{M}-72]^+$  ion corresponding to the loss of an EtOCO-group, whereas the minor peaks were  $[\text{M}]^+$  and  $[\text{M}-44; \text{M}-\text{OEt}]^+$  ions. For five di-EOC-PFP derivatives of catechol estrogens, the  $[\text{M}-72-72; \text{M}-2\text{EtOCO}]^+$  ion formed the base peak, whereas  $[\text{M}-72-44]^+$ ,  $[\text{M}-44-44]^+$ ,  $[\text{M}-72]^+$ ,  $[\text{M}-44]^+$ , 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 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 show 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



**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; peak 2, 3-MeO-E1; peak 3, 17 $\alpha$ -E2; peak 4, E3; peak 5, 17 $\beta$ -E2; peak 6, 17-epi-E3; peak 7, 16 $\alpha$ -OH-E1; peak 8, 16-epi-E3; peak 9, 4-MeO-E2; peak 10, E1; peak 11, 2-MeO-E2; peak 12, 16-keto-E2; peak 13, 4-MeO-E1; peak 14, 2-MeO-E1; peak 15, 2-OH-E3; peak 16, 4-OH-E2; peak 17, 2-OH-E2; peak 18, 4-OH-E1; and peak 19, 2-OH-E1. The sample was separated through a thermally stable MXT-1 capillary column (30 m  $\times$  0.25 mm I.D., 0.25  $\mu$ m 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 (from 5.50 to 6.30 min) was set at  $m/z$  300, 432, and 448 for the monitoring of 11 to 14; and 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.

estrogens showed low reaction yields of 11-45% by comparison with the addition of TEA (20  $\mu$ l) that represented 100% efficacy. This situation 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 of 10-40  $\mu$ l in 1 ml of a buffer solution was tested under the same reaction conditions (50  $\mu$ l 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  $\mu$ l, and 30  $\mu$ l of TEA was chosen because of its rapid evaporation rate.

In alkyloxycarbonylation, an alkaline condition is essential to promote the deprotonation of the reactive species (30-32, 34), and the pH range of 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 though 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% K<sub>2</sub>CO<sub>3</sub> solution. To set an optimal volume of ECF, the amount of ECF was also tested with volumes of 30-200  $\mu$ l in 1 ml of aqueous media at pH 8. The highest reaction yield was obtained in the range of 50-200  $\mu$ l ECF. Hence, 50  $\mu$ l of ECF was used in this study. Further experiments were carried out to optimize the reaction time. The experiment involved increasing the time

(from 10 s to 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 with vortexing at 25°C in a few seconds. Therefore, vortexing for 30 s was chosen as the simplest condition.

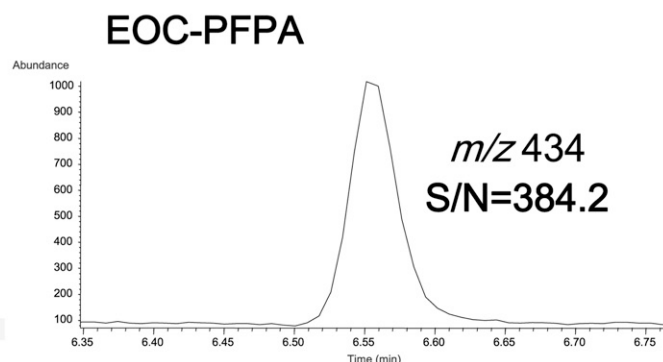
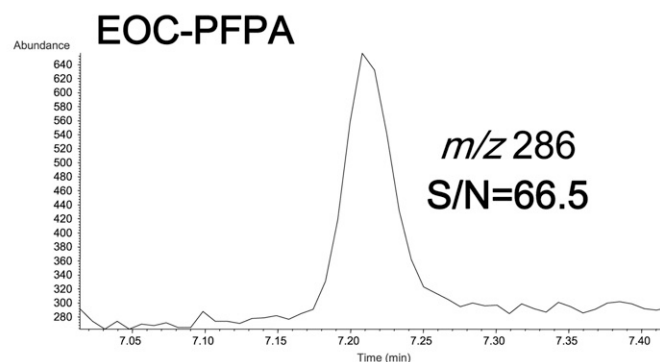
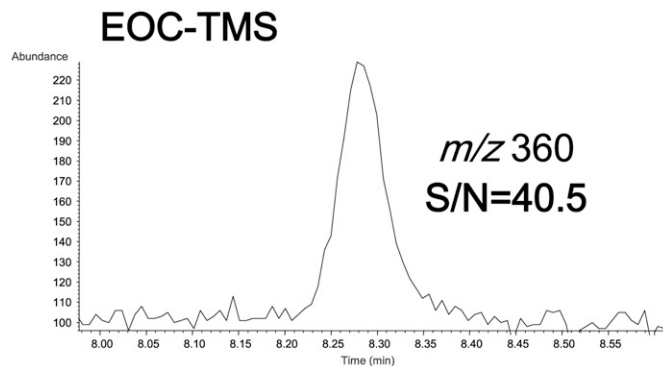
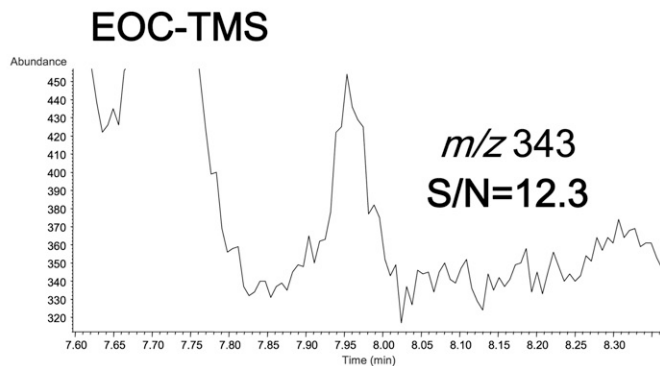
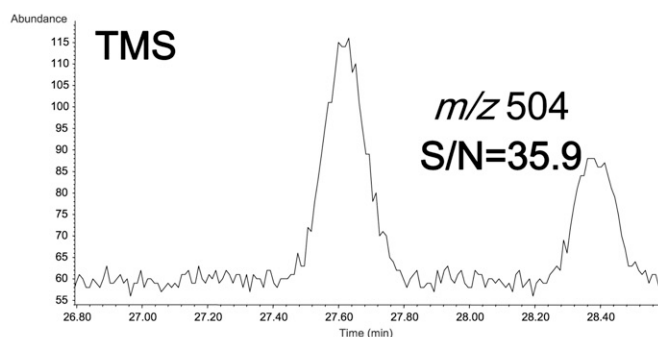
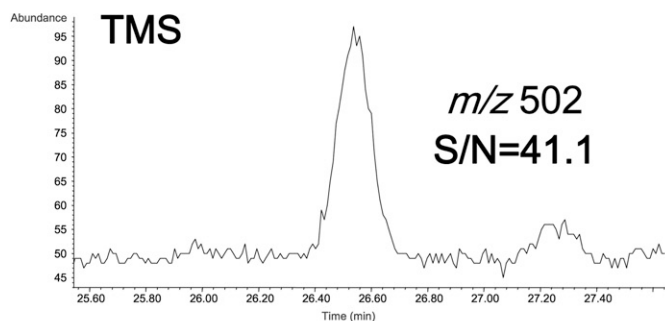
The sample pH did not affect extraction efficiency, and extraction was performed without additional pH adjustment after ethoxycarbonylation. As the extraction solvents, five different organic solvents, EA, MTBE, a mixture of EA and *n*-hexane (2:3, v/v), *n*-pentane, and *n*-hexane, were tested to assess the enhanced extraction efficiency and reduced matrix interference. The order of diminishing matrix noise was *n*-hexane  $\approx$  *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 was 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 accuracy, precision, selectivity, sensitivity, reproducibility, and stability using spiked samples prepared with the estrogen-free urine.

## 2-OH-E1

## 2-OH-E2



**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.

The LOD and LOQ, which are sample concentrations required to give a higher S/N ratio > 3 and 10, respectively, were also evaluated (Table 2). The LOQs of the EOC-PFP derivatives with the estrone-type moiety ranged from 0.06 to ~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 be 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  $10^3$ -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 three different levels of low (0.1 or 0.5 ng/ml), medium (2 ng/ml), and high (10 ng/ml) concentration. The intraday ( $n = 5$ ) precision (expressed as % CV) ranged from 1.6 to 11.5%, whereas accuracies (expressed as % bias) ranged from 94.0 to 109.5%; interday ( $n = 5$ ) 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 Materials and Methods (see supplementary Table I). Both



TABLE 2. Validation results of the overall method in the intra- and interday assays

Compound Abbreviation	LOD <sup>a</sup>	LOQ <sup>b</sup>	Recovery <sup>c</sup>	Linearity <sup>d</sup>	Intraday (n=5)		Interday (n=5)	
					CV <sup>e</sup>	Accuracy <sup>e</sup>	CV	Accuracy
E1	0.04	0.08	95.2	0.9984	4.8	109.5	6.2	103.6
17β-E2	0.01	0.02	103.9	0.9997	1.6	109.2	1.4	107.7
E3	0.01	0.04	75.3	0.9974	4.7	106.3	8.1	99.4
2-OH-E1	0.05	0.10	90.8	0.9953	11.5	101.5	10.5	100.6
2-OH-E2	0.01	0.02	96.7	0.9978	4.0	99.7	10.1	103.5
4-OH-E1	0.05	0.10	80.7	0.9972	11.6	97.7	11.3	98.9
4-OH-E2	0.01	0.04	93.3	0.9985	6.8	97.9	9.4	106.7
2-MeO-E1	0.02	0.04	92.9	0.9980	7.9	105.4	7.6	99.3
2-MeO-E2	0.01	0.02	103.8	0.9997	4.1	98.7	2.8	98.9
3-MeO-E1	0.05	0.10	86.6	0.9977	10.9	100.0	9.4	108.5
3-MeO-E2	0.01	0.02	95.0	0.9984	4.6	100.2	7.5	102.5
4-MeO-E1	0.04	0.10	98.7	0.9976	5.6	107.9	6.7	100.1
4-MeO-E2	0.01	0.02	102.3	0.9996	2.3	106.8	2.9	100.3
17-epi-E3	0.01	0.04	107.0	0.9994	7.1	101.3	7.8	98.4
16-epi-E3	0.01	0.04	109.4	0.9993	6.1	101.5	6.8	97.4
17α-E2	0.02	0.04	109.5	0.9994	3.4	96.3	2.9	100.4
16α-OH-E1	0.02	0.06	100.1	0.9986	9.2	108.9	8.6	102.2
16-keto-E2	0.02	0.06	102.7	0.9981	6.9	94.0	8.9	91.9
2-OH-E3	0.10	0.50	48.7	0.9950	24.8	102.2	17.4	104.3

<sup>a</sup>The limit of detection was measured at S/N ratio > 3.

<sup>b</sup>Limit of quantification was measured at S/N ratio > 10.

<sup>c</sup>Absolute recoveries were calculated by comparing the peak height ratios of the samples using the described method with those of their nonextracted counterparts from three different QC concentrations in five replicates.

<sup>d</sup>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 five different days.

<sup>e</sup>Precision and accuracy were expressed as the mean values of data obtained from three QC samples through intra- and interday assays.

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 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% relative standard deviation, 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 freezing and thawing cycles did not appear to affect the concentration of estrogens. Instability can occur not only 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 re-analyzed in the case of instrument 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 (< ±12% RSD).

#### Application to postmenopausal women with osteoporosis

The usefulness of this novel method for quantifying low levels of endogenous estrogen 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 eight estrogens with a total of above 90% incidence, E1 was the most abundant, 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

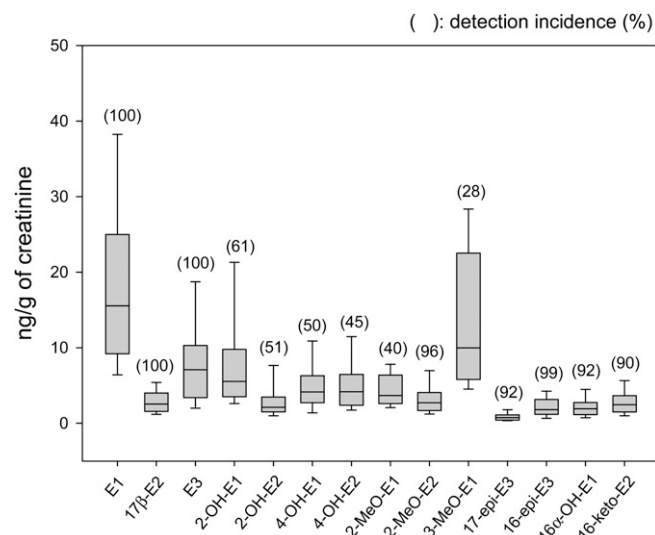


Fig. 3. Urinary estrogen levels obtained from 100 postmenopausal female patients with osteoporosis. All quantitative data was corrected by the urinary creatinine values.

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), TMS derivatives are not sensitive enough for quantification of urinary estrogen metabolites and the derivatization process is time consuming. As a different approach, the direct derivatization in aqueous solution to make estrogen extractable by nonpolar organic solvents was more preferred. With extractive two-phase EOC 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 EOC with less bulky methyl or ethyl chloroformate, with subsequent perfluoroacylation or alkylsilylation of the remaining active hydrogen atoms. However, attempts have rarely been made to adopt this method for biomolecule analysis.

In our previous study, a high-temperature, stainless steel capillary column was successfully used for the analysis of lipid molecules as their TMS derivatives with good chromatographic resolution (36, 39), while fused-silica capillary columns had poor chromatographic properties or long retention times. Initial studies with a fused-silica capillary column above 300°C produced poor results because the fused-silica capillary column become brittle over time at above 300°C, and all analytes were eluted as broad peaks accompanied by extraneous peaks. To continue our 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 thermostability resulted in better detectability.

Using the EOC-PFP derivatization, 16 $\alpha$ -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 similar molecular weight and mass fragments. In general, 16 $\alpha$ -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 (16-epi-E3, 16 $\alpha$ -OH-E1, and 4-MeO-E2) were co-eluted using 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). These methods are capable of quantitatively measuring 15 estrogen metabolites and have been applied successfully to urine samples from healthy premenopausal and postmenopausal women. However, there are limitations, including a long analytical time (70 min) for the separation of the 15 estrogens, which might not be effective in large-scale applications. In our 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, 17 $\beta$ -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, 17 $\beta$ -E2, E3, 2-OH-E1, and 16-keto-E2) of these 15 estrogens were determined to be abundant estrogens in both groups (20, 25). 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 have been measured using immunoassay kits and GC-MS. However, 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 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 down to pg/ml levels of urinary estrogen metabolites. The good sensitivity of this method even allowed measurements of the estrogen metabolites in the urine of postmenopausal female patients with osteoporosis. This can be a useful technique for a clinical diagnosis and as a mining biomarker in estrogen-related disorders. ■■

## REFERENCES

1. Im, A., V. G. Vogel, G. Ahrendt, S. Lloyd, C. Ragin, S. Garte, and E. Taioli. 2009. Urinary estrogen metabolites in women at high risk for breast cancer. *Carcinogenesis*. **30**: 1532–1535.
2. Lépine, J., E. Audet-Walsh, J. Grégoire, B. Têtu, M. Plante, V. Ménard, P. Ayotte, J. Brisson, P. Caron, L. Villeneuve, et al. 2010. Circulating estrogens in endometrial cancer cases and their relationship with tissular expression of key estrogen biosynthesis and metabolic pathways. *J. Clin. Endocrinol. Metab.* **95**: 2689–2698.
3. Rodriguez, C., A. V. Patel, E. E. Calle, E. J. Jacob, and M. J. Thun. 2001. Estrogen replacement therapy and ovarian cancer mortality in a large prospective study of US women. *JAMA*. **285**: 1460–1465.
4. Lee, S. H., K. M. Kim, B. H. Jung, W. Y. Chung, C. S. Park, and B. C. Chung. 2003. Estrogens in female thyroid cancer: alteration of urinary profiles in pre- and post-operative cases. *Cancer Lett.* **189**: 27–32.
5. Carruba, G. 2006. Estrogens and mechanisms of prostate cancer progression. *Ann. N. Y. Acad. Sci.* **1089**: 201–217.
6. Khosla, S. 2010. Update on estrogens and the skeleton. *J. Clin. Endocrinol. Metab.* **95**: 3569–3577.
7. Zhu, B. T., and A. H. Conney. 1998. Functional role of estrogen metabolism in target cells: review and perspectives. *Carcinogenesis*. **19**: 1–27.
8. Zhu, B. T., and A. J. Lee. 2005. NADPH-dependent metabolism of 17 $\beta$ -estradiol and estrone to polar and nonpolar metabolites



- by human tissues and cytochrome P450 isoforms. *Steroids*. **70**: 225–244.
9. Dawling, S., N. Roodi, R. L. Mernaugh, X. Wang, and F. F. Parl. 2001. Catechol-O-methyltransferase (COMT)-mediated metabolism of catechol estrogens: comparison of wild-type and variant COMT isoforms. *Cancer Res*. **61**: 6716–6722.
  10. Fishman, J., and C. Martucci. 1980. Biological properties of 16 alpha-hydroxyestrone: implications in estrogen physiology and pathophysiology. *J. Clin. Endocrinol. Metab*. **51**: 611–615.
  11. Liehr, J. G. 2000. Is estradiol a genotoxic mutagenic carcinogen? *Endocr. Rev*. **21**: 40–54.
  12. Yager, J. D., and N. E. Davidson. 2006. Estrogen carcinogenesis in breast cancer. *N. Engl. J. Med*. **354**: 270–282.
  13. Korhonen, M. K., K. O. Juntunen, and U. H. Stenman. 1980. Enzyme immunoassay of estriol in pregnancy urine. *Clin. Chem*. **26**: 1829–1831.
  14. McGuinness, B. J., M. J. Power, and P. F. Fottrell. 1994. Radioimmunoassay of 2-hydroxyestrone in urine. *Clin. Chem*. **40**: 80–85.
  15. Moon, J-Y., H-J. Jung, M. H. Moon, B. C. Chung, and M. H. Choi. 2009. Heat-map visualization of gas chromatography-mass spectrometry based quantitative signatures on steroid metabolism. *J. Am. Soc. Mass Spectrom*. **20**: 1626–1637.
  16. Ha, Y. W., J-Y. Moon, H-J. Jung, B. C. Chung, and M. H. Choi. 2009. Evaluation of plasma enzyme activities using gas chromatography-mass spectrometry based steroid signatures. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci*. **877**: 4125–4132.
  17. Choi, M. H., K. R. Kim, and B. C. Chung. 2000. Determination of estrone and 17 $\beta$ -estradiol in human hair by gas chromatography-mass spectrometry. *Analyst (Lond.)*. **125**: 711–714.
  18. Courant, F., L. Aksglaede, J. P. Antignac, F. Monteau, K. Sorensen, A. M. Andersson, N. E. Skakkebaek, A. Juul, and B. L. Bizec. 2010. Assessment of circulating sex steroid levels in prepubertal and pubertal boys and girls by a novel ultrasensitive gas chromatography-tandem mass spectrometry method. *J. Clin. Endocrinol. Metab*. **95**: 82–92.
  19. Yamashita, K., M. Okuyama, Y. Watanabe, S. Honma, S. Kobayashi, and M. Numazawa. 2007. Highly sensitive determination of estrone and estradiol in human serum by liquid chromatography-electrospray ionization tandem mass spectrometry. *Steroids*. **72**: 819–827.
  20. Falk, R. T., X. Xu, L. Keefer, T. D. Veenstra, and R. G. Ziegler. 2008. A liquid chromatography-mass spectrometry method for the simultaneous measurement of 15 urinary estrogens and estrogen metabolites: assay reproducibility and interindividual variability. *Cancer Epidemiol. Biomarkers Prev*. **17**: 3411–3418.
  21. Penning, T. M., S. H. Lee, Y. Jin, A. Gutierrez, and I. A. Blair. 2010. Liquid chromatography-mass spectrometry (LC-MS) of steroid hormone metabolites and its applications. *J. Steroid Biochem. Mol. Biol*. **121**: 546–555.
  22. Stanczyk, F. Z., M. M. Cho, D. B. Endres, J. L. Morrison, S. Patel, and R. J. Paulson. 2003. Limitations of direct estradiol and testosterone immunoassay kits. *Steroids*. **68**: 1173–1178.
  23. Hsing, A. W., F. Z. Stanczyk, A. Bélanger, P. Schroeder, L. Chang, R. T. Falk, and T. R. Fears. 2007. Reproducibility of serum sex steroid assays in men by RIA and mass spectrometry. *Cancer Epidemiol. Biomarkers Prev*. **16**: 1004–1008.
  24. Santen, R. J., J. S. Lee, S. Wang, L. M. Demers, N. Mauras, H. Wang, and R. Singh. 2008. Potential role of ultra-sensitive estradiol assays in estimating the risk of breast cancer and fractures. *Steroids*. **73**: 1318–1321.
  25. Faupel-Badger, J. M., B. J. Fuhrman, X. Xu, R. T. Falk, L. K. Keefer, T. D. Veenstra, R. N. Hoover, and R. G. Ziegler. 2010. Comparison of liquid chromatography-tandem mass spectrometry, RIA, and ELISA methods for measurement of urinary estrogens. *Cancer Epidemiol. Biomarkers Prev*. **19**: 292–300.
  26. Blair, I. A. 2010. Analysis of estrogens in serum and plasma from postmenopausal women: past present, and future. *Steroids*. **75**: 297–306.
  27. Nishio, T., T. Higashi, A. Funashi, J. Tanaka, and K. Shimada. 2007. Development and application of electrospray-active derivatization reagents for hydroxysteroids. *J. Pharm. Biomed. Anal.* **44**: 786–795.
  28. Yang, W. C., F. E. Regnier, D. Sliva, and J. Adamec. 2008. Stable isotope-coded quaternization for comparative quantification of estrogen metabolites by high-performance liquid chromatography-electrospray ionization mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci*. **870**: 233–240.
  29. Xu, X., L. K. Keefer, R. G. Ziegler, and T. D. Veenstra. 2007. A liquid chromatography-mass spectrometry method for the quantitative analysis of urinary endogenous estrogen metabolites. *Nat. Protoc*. **2**: 1350–1355.
  30. Hušek, P. 1998. Chloroformates in gas chromatography as general purpose derivatizing agents. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci*. **717**: 57–91.
  31. Choi, M. H., K. R. Kim, and B. C. Chung. 2000. Determination of hair polyamines as N-ethoxycarbonyl-N-pentafluoropropionyl derivatives by gas chromatography-mass spectrometry. *J. Chromatogr. A*. **897**: 295–305.
  32. Choi, M. H., and B. C. Chung. 2001. N-isobutyloxycarbonylation for improved detection of 3'-hydroxystanozolol and its 17-epimer in doping control. *Analyst*. **126**: 306–309.
  33. Haberhauer-Troyer, C., G. Alvarez-Llamas, E. Zitting, P. Rodríguez-González, E. Rosenberg, and A. Sanz-Medel. 2003. Comparison of different chloroformates for the derivatisation of seleno amino acids for gas chromatographic analysis. *J. Chromatogr. A*. **1015**: 1–10.
  34. Tao, X., Y. Liu, Y. Wang, Y. Qiu, J. Lin, A. Zhao, M. Su, and W. Jia. 2008. C-MS with ethyl chloroformate derivatization for comprehensive analysis of metabolites in serum and its application to human uremia. *Anal. Bioanal. Chem*. **391**: 2881–2889.
  35. dos Santos Pereira, A., M. C. Padilha, and F. Radler de Aquino Neto. 2004. Two decades of high temperature gas chromatography (1983–2003): what's next? *Microchem. J*. **77**: 141–149.
  36. Jung, H-J., W-Y. Lee, B. C. Chung, and M. H. Choi. 2009. Mass spectrometric profiling of saturated fatty acid esters of steroids separated by high-temperature gas chromatography. *J. Chromatogr. A*. **1216**: 1463–1468.
  37. Husdan, H., and A. Rapoport. 1968. Estimation of creatinine by the Jaffe reaction. A comparison of three methods. *Clin. Chem*. **14**: 222–238.
  38. Choi, M. H., K. R. Kim, and B. C. Chung. 2000. Simultaneous determination of urinary androgen glucuronides by high temperature gas chromatography-mass spectrometry with selected ion monitoring. *Steroids*. **65**: 54–59.
  39. Jung, H-J., W-Y. Lee, Y. S. Yoo, B. C. Chung, and M. H. Choi. 2010. Database-dependent metabolite profiling focused on steroid and fatty acid derivatives using high-temperature gas chromatography-mass spectrometry. *Clin. Chim. Acta*. **411**: 818–824.
  40. Parl, F. F., S. Dawling, N. Roodi, and P. S. Crooke. 2009. Estrogen metabolism and breast cancer: a risk model. *Ann. N. Y. Acad. Sci*. **1155**: 68–75.