Brazilian Journal of Medical and Biological Research (2015) 48(2): 146-153, http://dx.doi.org/10.1590/1414-431X20144129
ISSN 1414-431X

Measurement of serum estrogen and estrogen metabolites in pre- and postmenopausal women with osteoarthritis using high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry

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Abstract

Although 17β-estradiol (E_2) deficiency has been linked to the development of osteoarthritis (OA) in middle-aged women, there are few studies relating other estrogens and estrogen metabolites (EMs) to this condition. We developed a high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) method to measure the levels of six EMs (i.e., estrone, E_2, estriol, 2-hydroxyestrone, 2-hydroxyestradiol, and 16a-hydroxyestrone) in healthy pre- and postmenopausal women and women with OA. This method had a precision ranging from 1.1 to 3.1% and a detection limit ranging from 10 to 15 pg. Compared to healthy women, serum-free E_2 was lower in the luteal and postmenopausal phases in women with OA, and total serum E_2 was lower in postmenopausal women with OA. Moreover, compared to healthy women, total serum 2-hydroxyestradiol was higher in postmenopausal women with OA and total serum 2-hydroxyestrone was lower in both the luteal and follicular phases in women with OA. In conclusion, our HPLC-ESI-MS/MS method allowed the measurement of multiple biochemical targets in a single assay, and, given its increased cost-effectiveness, simplicity, and speed relative to previous methods, this method is suitable for clinical studies.

Key words: Estrogen; Estrogen metabolites; Menopause; Serum; Osteoathritis

Introduction

Estrogens are endogenous female hormones required for the growth and development of target tissues, including the mammary gland, and which play a major role in the etiology of breast cancer (1,2). Estrogen metabolism has been related to osteoporosis and increased fracture risk (3,4), and lower baseline serum 17β-estradiol (E_2) and urinary 2-hydroxyestrone (2-OHE_1) levels have been linked to knee osteoarthritis (OA) in middle-aged women (5). That said, there are relatively few studies linking serum levels of other estrogens and estrogen metabolites (EMs) to the pathogenesis of female OA.

Endogenous estrones (E_1) and E_2 are synthesized from the androgenic precursors androstenedione and testosterone, respectively, in a reaction catalyzed by cytochrome P450 aromatase, which is expressed primarily in ovarian granulosa cells, adipose stromal cells, and the placenta. Circulating E_2, the primary estrogen in premenopausal and early perimenopausal women, declines with menopause. E_1 generated by oxidation of E_2 is hydroxylated via two mutually exclusive pathways (6) to generate either 2-hydroxyestrone (2-OHE_1) or 16a-hydroxyestrone (16a-OHE_1; Figure 1), which are estrogen agonists (7) with target tissue-specific biological activities distinct from one another and from E_2. Compared to E_2, 16a-OHE_1 binds with lower affinity to both the estrogen receptor (8) and to serum sex hormone-binding globulin, and is consequently

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Received May 15, 2014. Accepted September 9, 2014. First published online November 14, 2014.

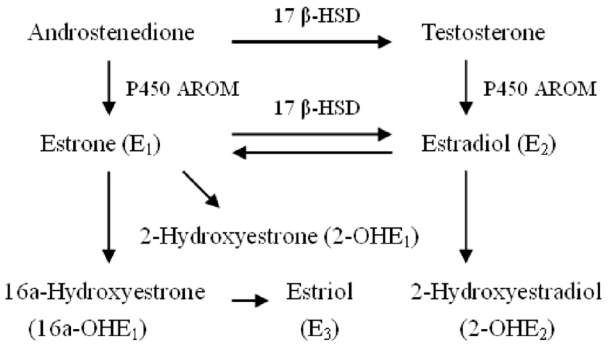

Figure 1. Estrogen metabolism pathways, including estrone, estradiol, and estrogen metabolites. 17β-HSD: 17beta-hydroxysteroid dehydrogenase; P450 AROM: cytochrome P450 aromatase.

more available in estrogen-sensitive target tissues than E_2. Although 2-hydroxyestrogens have a reduced binding affinity for the estrogen receptor compared to 16a-OHE_1, they participate in oxidation/reduction reactions (9,10).

A variety of analytical methods have been developed to analyze EMs, including spectrophotometry (11) and thin-layer chromatography (12) for high EM levels, and radioimmunoassay (RIA) (13,14) and enzyme-linked immunosorbent assay (15) for trace EM levels. Given the problems of cross-contamination associated with these techniques, however, gas chromatography (GC) (16) and high-performance liquid chromatography (HPLC) (17,18) are typically used to detect ingredients in Chinese formulated products because of their outstanding separation power and automatable nature. However, owing to the presence of multiple components in blood samples, EMs cannot be readily quantified using GC and HPLC, which discriminate by retention time only.

HPLC-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) offers a variety of advantages over these methods for the quantification of serum EMs. Chromatographic separation removes most of the interferences caused by impurities, and multiple co-eluting moieties with distinct molecular masses and chemical structures generate characteristic fragments and specific mass spectra. Although LC/MS methods (19-21) measure more biochemical targets in a single assay than HPLC-ESI-MS/MS, the multiple reaction-monitoring quantitative model of HPLC-ESI-MS/MS allows the identification and quantification of products in the picogram range in only 10 mL of blood. We established an HPLC-ESI-MS/MS method to determine serum levels of 6 EMs in pre- and postmenopausal women with OA and in healthy women (Figure 2).

Material and Methods

Equipment

The following equipment was used: TI-H-15 supersonic generator (ELMA, Germany), incubator (LRH-250A, Guangdong Province Medical Instrument Factory, China), LABORTA 4003 rotary evaporation (Heidolphon, Germany), U3 aqueous bath (Julabo, Germany), LRH-250A homeothermia incubator (Medical Apparatus and Instruments Factory, China), Mili-Q deionized water deviser (Millipore, USA), ME-5 electronic balance (Sartorius, Germany), API3000 tandem quadrupole axle mass spectrometer (AB Company, USA), and an Agilent 1100 liquid chromatography system (Agilent, USA), ZORBAX C18 chromatographic column (4.6 × 250 mm, 5 μm; SN: USCN004202; Agilent).

Reagents

Chlormycetin, glucuronidase, and aryl vitriol acetum enzyme were obtained from Sigma Chemical Co. (USA). All selected EMs and chlormycetin had chemical purity ≥98% and were applied without further purification. Methanol, acetonitrile, toluene (chromatographic-grade purity), glucuronidase/aryl vitriol acetum enzyme solution, ammonium acetate, acetic acid, methyl tertiary butyl ether (MTBE), and acetidin were obtained from Merck & Co., Inc. (USA).

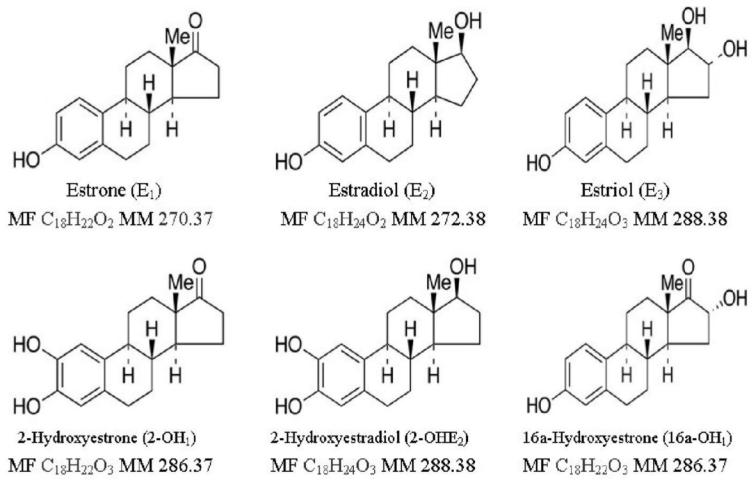

Figure 2. Chemical structure, molecular formula (MF), and molecular mass (MM) of the 6 estrogen metabolites.

Preparation of standard solutions

Stock solutions of 6 EMs (Figure 2) and chlormycetin were each prepared at 80 μg/mL by dissolving 2 mg of each EM or chlormycetin powder in methanol to a final volume of 25 mL in a volumetric flask. Stock solutions were monitored for time-dependent degradation by measuring the absolute peak height of each EM and chlormycetin. The stock solutions were stable for at least 50 days if stored at –20°C. Standard solutions were prepared by diluting 1.0 mL of each EM stock solution in methanol to a final volume of 100 mL.

Calibration standards (1, 5, 10, 20, 40, and 80 ng/mL) were prepared by diluting standard solutions in methanol. Internal standard solutions were prepared by diluting 0.25 mL of chlormycetin stock standard solution in methanol to a final volume of 20 mL.

Acetate buffer solution preparation

Acetate buffer solution at 0.04 M was prepared by dissolving 43.0 g ammonium acetate in 200 mL water, and then adding 25.2 g glacial acetic acid. The pH was adjusted to 5.0 and the final volume was brought to 1000 mL using water.

Subjects

All subjects were Asian. Baseline characteristics included age, disease duration, body height, body weight, and body mass index (Table 1). Thirty-two premenopausal outpatients (aged 37-47 years) and 32 postmenopausal inpatients (aged 55-68 years) with OA of the knee were selected as the experimental groups, and 48 healthy women, including 24 premenopausal women (aged 37-46 years) and 24 postmenopausal women (aged 56-69 years), participated as the control group. The length of time since menopause in all experimental and control postmenopausal women was in excess of 5 years. All patients were from the Linyi People's Hospital (Shandong, China). OA patients were from the Orthopedics Department, and healthy controls were from the Medical Examination Center. All subjects gave written informed consent and the study was approved by the Institute Ethics Committee of Linyi People's Hospital.

Patients reporting knee pain and stiffness (time <30 min) or crepitus were diagnosed as symptomatic OA if a definite osteophyte was observed in the symptomatic knee on plain radiographs, in accordance with American College of Rheumatology criteria for OA (22). Exclusion criteria for patients included: nonsteroidal anti-inflammatory drugs 3 days prior to the investigation; corticosteroid therapy during the 6 months prior to the investigation; use of contraceptives, weight-reducing aids, vitamin D, or any hormone-replacement therapy; recent history of medication, malignancy, hypertension, diabetes, viral hepatitis, parenchyma liver diseases, or fatty liver. Exclusion criteria for healthy pre- or postmenopausal women included a history of OA, smoking and alcohol use, and current pregnancy.

Blood sample collection

Venous blood was collected from premenopausal and postmenopausal OA patients and healthy women. In premenopausal subjects, blood samples were collected during the follicular (days 5-7) and luteal (days 22-25) phases of the same menstrual cycle, whereas only one blood sample was drawn from postmenopausal subjects. Menstrual and menopausal status was self-reported. Menopausal status was defined as premenopausal (women having regular menses) or postmenopausal (no menses in the last 12 months). Blood was allowed to clot for 1 h at room temperature, then centrifuged at 300 *g* for 10 min and frozen at –80°C until assayed.

Sample preparation procedure

Internal standard solutions (50 μL) were added to a 0.5-mL aliquot of serum, after which 100 μL freshly prepared glucuronidase/sulfatase and 2.0 mL 0.04 M acetate buffer solutions were added. After vortexing, the sample was transferred to an incubator and hydrolyzed at 37°C for 4-8 h. After hydrolysis, 5 mL acetidin was added to the mixture, which was then placed in the supersonic generator for 5 min. After centrifuging at 12,000 *g* for 10 min, the supernatant (organic liquid phase) was transferred to a rotary evaporation bottle, and the remaining solution was extracted with acetidin. The supernatant was then mixed and evaporated to dryness in a rotary evaporation apparatus at 40°C, after which 0.5 mL methanol was added to dissolve the extract. Samples were analyzed by HPLC-ESI-MS/MS after 0.45-μm filtering (Millipore). Samples for free serum

Table 1. Baseline characteristics of subjects.

	Follicular phase		Luteal phase		Postmenopausal phase	
	OA (n = 32)	Control (n = 24)	OA (n = 32)	Control (n = 24)	OA (n = 32)	Control (n = 32)
Age (years)	41.8 ± 5.7	43.4 ± 7.4	41.8 ± 5.7	43.4 ± 7.4	60.6 ± 6.4	56.9 ± 3.0
Disease duration	4.6 ± 2.0	NA	4.6 ± 2.0	NA	16.3 ± 2.2	NA
Body height (cm)	156.2 ± 0.7	151.2 ± 0.6	156.2 ± 0.7	151.2 ± 0.6	154.8 ± 0.6	159.1 ± 0.8
Body weight (kg)	59.2 ± 5.4	56.7 ± 6.4	59.2 ± 5.4	56.7 ± 6.4	71.5 ± 4.9	69.1 ± 6.5
BMI (kg/m^2)	24.2 ± 1.7	23.0 ± 1.8	24.2 ± 1.7	23.0 ± 1.8	30.5 ± 1.4	27.6 ± 3.4

Data are reported as means ± SD. OA: osteoarthritis; control: healthy women; BMI: body mass index; NA: not applicable.

Table 2. Mass spectrometry parameters of HPLC-ESI-MS/MS measurements of estrogen metabolites and chlormycetin.

	Q1/Q3	DP	FP	EP	CE	CXP
E_1	269/145	121	219	11	50	34
E_2	271/183	30	200	10	55	15
E_3	287/145	121	350	10	60	21
2-OHE_1	285/160	121	219	11	52	15
2-OHE_2	287/147	121	219	11	60	15
16a-OHE_1	285/145	85	350	10	54	23
Chlormycetin	320/257	30	300	10	21	15

Q1/Q3: parent ion/daughter ion; DP: declustering potential; FP: focusing potential; EP: entrance potential; CE: collision energy; CXP: collision cell exit potential. E_1: estrone; E_2: estradiol; E_3: estriol; 2-OHE_1: 2-hydroxyestrone; 2-OHE_2: 2-hydroxyestradiol; 16a-OHE_1: 16a-hydroxyestrone.

EM measurement were processed as described earlier, with the exclusion of the glucuronidase/sulfatase hydrolysis step.

Calibration standards preprocessing

Internal standard solutions (50 µL) were added to each of the calibration standards (1, 5, 10, 20, 40, and 80 ng/mL), following the addition of 100 µL freshly prepared glucuronidase/sulfatase and 2.0 mL 0.04-M acetate buffer solution. After vortexing, the samples were transferred to an incubator at 37°C for 4 h.

HPLC-ESI-MS/MS analysis

HPLC-ESI-MS/MS analysis was performed using an API3000 tandem quadrupole axle mass spectrometer with a Turbo spray ESI ionization source. Data were processed using an Analyst 1.4 workstation. HPLC samples (10 µL) were injected on a ZORBAX C18 chromatographic column,

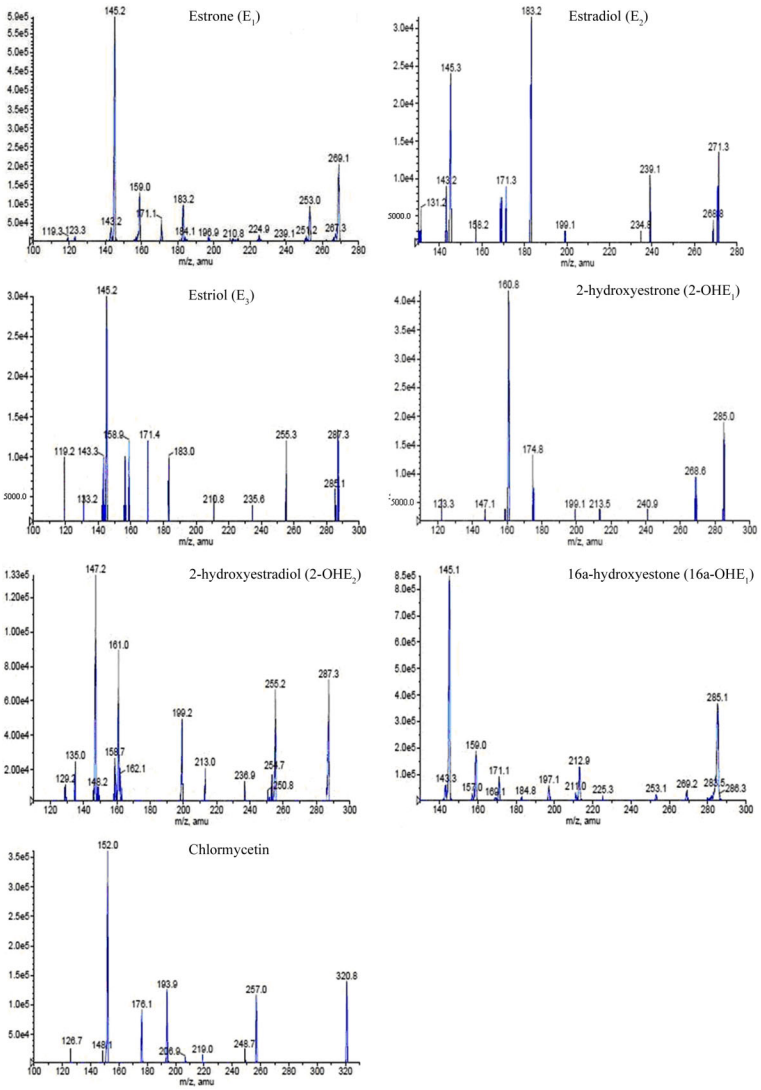

Figure 3. Product mass spectrum of EMs and internal standards. E_1: estrone; E_2: estradiol; E_3: estriol; 2-OHE_1: 2-hydroxyestrone; 2-OHE_2: 2-hydroxyestradiol; 16a-OHE_1: 16a-hydroxyestrone; chlormycetin.

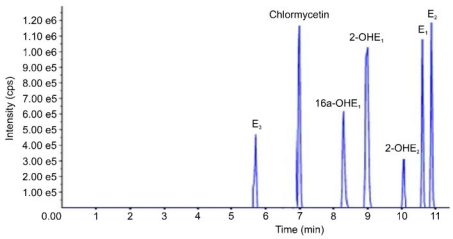

Figure 4. Total ion flow chart. E_1: estrone; E_2: estradiol; E_3: estriol; 2-OHE_1: 2-hydroxyestrone; 2-OHE_2: 2-hydroxyestradiol; 16a-OHE_1: 16a-hydroxyestrone.

and maintained at 35°C. The mobile phase consisted of methyl cyanide solution and operated at a flow rate of 200 μL/min. For the analysis of EMs, a linear gradient was established that changed the methyl cyanide/water solvent ratio from 20/7 to 20/13 over 15 min. The ionizing voltage was –4200 V in the negative ion mode, the ion pair of the parent ion (Q1)/daughter ion (Q3) was set up as the unit of discernment, and the dwell time of each ion pair was 150 ms. Other parameters are listed in Table 2.

Statistical analysis

Normal distributions were validated using the Kolmogorov-Smirnov method. Intergroup comparisons were carried out using an independent-samples *t*-test to determine whether experimental and control values in OA and healthy women were significantly different. All statistical analyses were performed using the SPSS software (USA). Type I error was assessed based on two-sided tests. P values less than 0.05 were considered to be significant.

Results

Characteristic ion pair options of MS/MS

For the phenolic hydroxyl group of estrogens as proton donors, the signal is known to be more sensitive in the negative ion mode than in the positive ion mode. In this study, an exteriorized needle pump mode was used for each standard solution of E_1, E_2, E_3, 2-OHE_1, 2-OHE_2, 16a-OHE_1, or chlormycetin. In negative ion mode, mass spectra were typical and specific for each reference substance (Figure 3).

Selection of color spectrum behavior and diagnosis ions

The most sensitive and characteristic parent ion and daughter ion of each reference substance were selected to compose an ion pair, which were then measured after separating with methyl cyanide solution. We found that a fixed-proportion mobile phase was unsuitable for quantitative analysis because it failed to separate the 6 EM reference substances effectively owing to the width of the chromatographic peaks. We subsequently established chromatographic conditions in which each reference substance was completely separated with satisfactory signal intensity (Figure 4).

Selection of extraction solvent

We tested the extraction efficiency of alcohol, acetone, ethylether, acetidin, MTBE, and chloroform with respect to the selected EMs and chlormycetin, and found that MTBE was the most efficient (data not shown).

Methodology index analysis

The precision, recovery, and other methodology indices for each ion pair are listed in Table 3. The detection limit was defined as the concentration corresponding to a level 10 times above the level of acoustic noise when a blank sample was added to each progressively diluted reference substance solution. Precision was measured from the relative standard deviation of the mean of 11 continuous measurements after adding 1.0 ng internal standard to each sample. Accuracy was defined as the mean value of each sample of 20 replicates with known concentrations, in which 1.0 ng internal standard was added. Recovery was calculated using the formula: $P\ (\%) = (C_{all} - C_{sample})/C_{addition} \times 100$.

Serum levels of selected estrogens and estrogen metabolites

Compared to healthy women, serum-free E_2 was lower ($P<0.05$) in the luteal and postmenopausal phases

Table 3. Methodology index of estrogen metabolite analyses.

	Linear range (ng/mL)	Linear equation	r	Precision (%)	Recovery (%)	Detection limit (pg)
E_1	0.2-10000	y = 0.01126 x – 0.01701	0.9994	2.6	98.8	10
E_2	0.2-10000	y = 0.00778 x + 0.00104	0.9998	1.3	103.4	10
E_3	0.7-10000	y = 0.00434 x – 0.00229	0.9997	2.1	97.6	15
2-OHE_1	1.0-10000	y = 0.00835 x + 0.03071	0.9995	1.8	107.4	10
2-OHE_2	0.5-10000	y = 0.00634 x + 0.03022	0.9999	1.1	99.4	15
16a-OHE_1	0.2-10000	y = 0.001132 x + 0.02173	0.9991	3.1	105.4	10

r: correlation coefficient; E_1: estrone; E_2: estradiol; E_3: estriol; 2-OHE_1: 2-hydroxyestrone; 2-OHE_2: 2-hydroxyestradiol; 16a-OHE_1: 16a-hydroxyestrone.

Table 4. Serum free (unconjugated) and total (conjugated + unconjugated) levels of selected estrogen metabolites in the follicular, luteal, and postmenopausal phases in women with OA and healthy controls.

	Follicular phase		Luteal phase		Postmenopausal phase	
	OA (n = 32)	Control (n = 24)	OA (n = 32)	Control (n = 24)	OA (n = 32)	Control (n = 24)
Free						
E_1	52.5 ± 21.7	54.1 ± 11.9	48.0 ± 6.6	51.3 ± 2.7	28.2 ± 9.4	31.9 ± 4.8
E_2	71.5 ± 14.2	75.1 ± 19.1	65.0 ± 9.2*	72.3 ± 3.2	13.7 ± 3.1*	18.2 ± 5.6
E_3	15.4 ± 4.8	14.7 ± 1.8	16.6 ± 2.0	17.4 ± 1.4	5.9 ± 1.4	7.0 ± 1.1
Total						
E_1	643.0 ± 310.5	670.9 ± 113.4	726.6 ± 42.0	756.5 ± 68.8	404.1 ± 62.5	426.6 ± 34.7
E_2	117.9 ± 14.5	123.8 ± 28.8	141.5 ± 9.3	149.4 ± 18.5	40.1 ± 8.0**	51.4 ± 12.1
E_3	48.0 ± 18.2	51.8 ± 11.6	49.5 ± 7.0	47.0 ± 6.2	24.7 ± 8.0	27.0 ± 5.9
2-OHE_1	262.7 ± 45.4*	304.1 ± 51.5	264.2 ± 40.4*	296.5 ± 31.1	64.1 ± 9.2	67.3 ± 8.4
2-OHE_2	27.1 ± 7.9	24.0 ± 7.8	26.3 ± 8.4	23.9 ± 7.2	16.9 ± 4.9**	11.6 ± 4.0
16a-OHE_1	23.1 ± 5.3	25.4 ± 6.7	22.1 ± 2.3	23.4 ± 1.6	11.6 ± 2.0	13.1 ± 1.2

Data are reported as means ± SD in pg/mL. OA: osteoarthritis; control: healthy women; E_1: estrone; E_2: estradiol; E_3: estriol; 2-OHE_1: 2-hydroxyestrone; 2-OHE_2: 2-hydroxyestradiol; 16a-OHE_1: 16a-hydroxyestrone. *$P<0.05$, OA compared to control; **$P<0.01$, OA compared to control (*t*-test).

in women with OA, and total serum E_2 was lower ($P<0.01$) in postmenopausal women with OA (Table 4). Furthermore, compared to healthy women, total serum 2-OHE_2 was higher ($P<0.01$) in postmenopausal women with OA and total serum 2-OHE_1 was lower ($P<0.05$) in both the luteal and follicular phases in women with OA. Neither free nor total serum levels of E_1 and E_3 in pre- and postmenopausal women with OA differed significantly from those in healthy subjects.

Discussion

OA involves the progressive degeneration of articular cartilage and structural changes in the underlying bone, including the development of marginal growths and osteophytes, and thickening of the periosteum (23). A century ago, the description by Cecil and Archer (24) of ''arthritis of the menopause,'' referring to the development of hand and knee OA during menopause, suggested that estrogen deficiency might play a role in OA. Subsequent epidemiological studies have indicated that the incidence and prevalence of hip, knee, and finger OA are greater in men than in women before 50 years of age, after which age they are increasingly more common in women compared to men (25).

The expression of estrogen receptors in articular cartilage (26) suggests that, in addition to estradiol deficiency, 2-OHE_1 and 2-OHE_2 might be involved in the etiology of female OA and that manipulation of their levels has potential in the prevention and cure of this condition. In this regard, future studies are required to determine the specific cell types, such as chondrocytes, synoviocytes, and bone cells, that are involved in reducing levels of 17β-estradiol (E_2) and 2-OHE_1, and in raising levels of 2-OHE_2.

Our HPLC-ESI-MS/MS assay had a detection limit of 10-15 pg/mL serum and a precision of 1.1-3.1%, enabling us to measure endogenous serum estrogens in pre- and postmenopausal women. The identification and quantification of serum estradiol levels in the low pre- and postmenopausal range (<30 pg/mL) are important prognostic tools for common chronic diseases of women (27). RIA methods are insufficiently accurate or sensitive to monitor serum estradiol at such low levels (28-30), and, although bioassays using recombinant yeast and HeLa cells are more sensitive than RIA, they lack specificity and convenience (31). Moreover, whereas GC/tandem mass spectrometry is sufficiently specific, sensitive, and accurate, it lacks the precision of our HPLC-ESI-MS/MS method (32-34). The MS-based detection of derivatized estrogens in multiple samples using the cation mode of analysis has been recently reported (35). Although this approach reportedly raised the sensitivity of analysis, the efficiency of derivation was not investigated in a previous study (36), suggesting that the derivation procedure might introduce errors. In addition, the isotope-labeled estrogens and EM standards used in previous studies (22,37,38) are more expensive than our negative ion mode MS/MS assay.

The free serum E_2 levels in pre- and postmenopausal women detected in the current study (Table 4) were statistically similar to those obtained previously (23), with four-fold lower standard deviations and a six-fold greater number of samples from postmenopausal women.

In conclusion, we have established an HPLC-ESI-MS/MS method for the simultaneous detection of serum levels of 6 EMs in pre- and postmenopausal women with OA and healthy controls. Because the MS/MS was performed

in multiple reaction-monitoring tandem quadrupole mass spectrometry using ESI in negative ion mode, blood samples derivatives were not necessary, making the assay more economical, rapid, and efficient. Accordingly, our HPLC-ESI-MS/MS method has potential clinical applications, as well as for treatment or intervention studies requiring precise measurements of fluctuations in EM levels, such as in perimenopausal women, or measurement of low analyte concentrations. In this regard, further improvements are required to minimize variability in measurements.

Acknowledgments

Research supported by the Excellent Young Scientist Research Award fund of Shandong China (#BS2011YY040), the Medical and Health Science and Technology Development Plan of Shandong China (#2011QW009), and the development projects of Shandong Province Science and Technology of Traditional Chinese Medicine (#2013ZDZK155).

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