Journal of Clinical Laboratory Analysis 13:266–272 (1999)

Combination of Automatic HPLC-RIA Method for Determination of Estrone and Estradiol in Serum

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We developed a highly sensitive assay for estrone and 17 β-estradiol in serum. Estrone and 17 β-estradiol, obtained by solid-phase extraction using a Sep pak tC18 cartridge, were purified by high-performance liquid chromatography (HPLC). Quantitation of estrone and 17 β-estradiol were carried out by radioimmunoassay. Not insignificantly, this automatic system of extraction and HPLC succeeded in analyzing 80 samples a week. Intra-assay coefficients of variation (CV) for estrone and 17 β-estradiol ranged from 19.5 to 28.7%, and from 8.5 to 13.7%, respectively. The minimum detectable dose for estrone and 17β-estradiol were 1.04 pg/ml and 0.64 pg/ml, respectively. The serum levels of 17 β-estradiol using our method strongly correlated with those by Gas chromatography mass spectrometry (GC-MS). The serum levels of estrone and 17 β-estradiol in 154 peri- and postmenopausal women were estimated to be between 15 and 27 pg/ml and between 3.5 and 24.0 pg/ml, respectively, while the serum level of 17 β-estradiol in postmenopausal women, in particular, was estimated to be from 3.5 to 6.3 pg/ml. For postmenopausal women who suffered from vasomotor symptoms, the mean levels of estrone and 17 β-estradiol at 12 to 18 hours after treatment with daily 0.625 mg conjugated equine estrogen (CEE) and 2.5 mg medroxyprogesterone acetate (MPA) were 135.0 and 21.3 pg/ml at 12 months, respectively. On the other hand, levels of estrone and 17 β-estradiol at 12 to 18 hours after treatment with CEE and MPA every other day, were 73.4 and 15.3 pg/ml, respectively. These highly sensitive assays for estrone and 17 β-estradiol are useful in measuring low levels of estrogen in postmenopausal women, and monitoring estrogen levels in women receiving CEE as hormone replacement therapy. J. Clin. Lab. Anal. 13:266–272, 1999.

Key words: estradiol; estrone; HPLC-RIA; postmenopause

INTRODUCTION

Direct radioimmunoassay (RIA) of serum 17 β-estradiol has generally been used for differential diagnosis of amenorrhea and for monitoring ovarian function in infertile patients (1,2). Determination of estradiol by direct RIA methods has proven to be convenient, but detection by such assays is limited to approximately 10 pg/ml, and cross-reactions with other steroid hormones cannot be eliminated. Furthermore, it has been reported that estimation of serum levels of 17 β-estradiol without extraction by RIA is affected by sex hormone binding globulin (SHBG) and unknown substances (3–5). Therefore, direct RIA methods are insufficient to evaluate low levels of 17 β-estradiol below 10 pg/ml. Recently, estrogen has played an important role in the preventative health care of postmenopausal women. The use of estrogens for the relief of vasomotor symptoms, improvement of vaginal discomfort due to atrophy (6), and the prevention of osteoporosis (7) and cardiovascular diseases (8) is now well established. It has also been reported that each tissue has a different sensitivity to estradiol (9,10). Therefore, it is necessary to monitor the serum level of estradiol in postmenopausal women receiving hormone therapy. Here, we describe the use of a highly sensitive HPLC-RIA system in the measurement of low levels of estrone and 17 β-estradiol in postmenopausal women and patients undergoing hormone replacement therapy.

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Received 6 April 1999; Accepted 13 July 1999

MATERIALS AND METHODS

Subjects

Blood samples were obtained from 20 healthy male volunteers aged 23 to 40, and 154 peri- and postmenopausal women aged 50 to 74. Five additional healthy postmenopausal volunteers aged 53 to 72 received a single oral administration of 0.625 mg conjugated equine estrogen (CEE, Premarin, Wyeth), and had blood samples taken for the first 3 hr, then at 6, 12, 24, 36, and 48 hr. Finally, 63 postmenopausal women aged 43 to 68 who suffered from vasomotor symptoms such as hot flush or atrophy of the vagina, were arbitrarily divided into two groups and treated with hormone replacement therapy. The first group (40 patients) received oral administration of 0.625 mg CEE and 2.5 mg medroxyprogesterone acetate (MPA, Provera, Upjohn) everyday, and the second group (23 patients) received oral administration of 0.625 mg CEE and 2.5 mg MPA every other day. In both groups, blood samples were drawn at 12 to 18 hr after medication. The blood was centrifuged at 4°C and the serum samples obtained were frozen until analysis.

Reagents

Progesterone, 17 α-hydroxyprogesterone, deoxycorticosterone, estrone, corticosterone, 17 β-estradiol, aldosterone, hydrocortisone, estriol and γ-globulin were purchased from Sigma Chemical Co. (St. Louis, MO). Testosterone was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Antiserum , ^{125}I-labeled reagents and precipitating reagent were obtained from Diagnostic System Laboratories (Texas). Tritium-labeled estrone and 17 β-estradiol, which were used after purification by HPLC, were obtained from Amersham International (Buckinghamshire, UK) and DuPont-NEN Research Products (Boston, MA), respectively. Other reagents were obtained from Kokusan Chemical Co. (Tokyo, Japan).

Extraction

To assess extraction efficiency, tritium-labeled estrone and 17 β-estradiol (approx. 2,000 dpm in 10 µl ethanol) were added to 1 ml of serum. After equilibration for about 30 min at room temperature, the serum was added to 1 ml of 0.1N HCl and shaken. The complete mixture was applied to an activated Sep pak tC18 PLUS cartridge (Waters Co., MA). After the cartridge was washed with 5 ml of 4% acetic acid and 5 ml of 50% methanol, steroid hormones were eluted with 3 ml of acetonitrile into glass tubes. The process of solid-phase extraction was automatically carried out by MilliLab Workstation (Waters Co., MA). The elution was evaporated at 40°C in a centrifugal concentrator. The residue was redissolved in a mixture (300 µl) of ethanol:*n*-hexane (1:4, v/v) and transferred to a vial for HPLC.

Chromatographic Apparatus and Conditions

The complete mixed sample was injected by WISP 710B autosampler (Waters Co., MA). HPLC was performed using a LC100P pump (Yokogawa Analytical System Inc., Tokyo, Japan) and an SPD-2A spectrophotometric detector (SHIMADZU Co., Kyoto, Japan). A CAPCELL PAK NH2 column (4.6 x 250 mm, 5 µm, Shiseido Co., Tokyo Japan) was used for separation of steroid hormones. The solvent composition was ethanol: *n*-hexane (1:4, v/v) at a flow rate of 1 ml/min. The retention time of estrone and 17 β-estradiol was calibrated by tritium-labeled compounds. Each fraction of estrone (8 to 10 min) and 17 β-estradiol (12 to 14 min) was collected by the Model 201 programmable fraction collector (GILSON Medical Electronics, Inc. Middleton, WI) and was evaporated in a centrifugal concentrator. The residue was redissolved in a 40 µl of ethanol and added to 760 µl of assay buffer (0.05 M phosphate buffer pH 7.4 containing 0.01% NaN_3, tween 20, 0.9% NaCl, and 0.2% γ-globulin).

Radioimmunoassay for Estrone and 17 β-Estradiol

From the fractions of reconstituted estrone and 17 β-estradiol, 20 µl of purified sample was transferred to a scintillation vial for recovery study. Radioactivity was measured in a 1,600 TR liquid scintillation counter (Packard Instruments Co. Inc., Meriden, CT). For radioimmunoassay of estrone, 200 µl of sample and various concentrations of standard estrone were pipetted into glass tubes (12 x 75 mm), and 100 µl of antibody against estrone and 10 µl of ^{125}I labeled estrone (approx. 5,000 cpm) was added to each tube, mixed and incubated at 4°C overnight. For the assay of estradiol, 10 µl of antibody against estradiol was added to the each tube which contained 200 µl of sample and various concentrations of standard estradiol, mixed and incubated at 4°C for 6 hr. Thereafter, ^{125}I labeled estradiol (approx. 5,000 cpm) was added and incubated at 4°C overnight. After incubation, 1 ml of precipitating reagent was added, vortexed and kept at room temperature for 15 min. The tubes were centrifuged at 1,500*g* for 20 min, and supernatants were decanted. The residues were counted by ARC-1000 gamma counter (ALOKA Co., Tokyo, Japan). The results of estrone and 17 β-estradiol concentrations were obtained using logit-log plot that was made by curve-fitting software (ALOKA RIA SYSTEM), and the following expression:

estrogen concentration (pg/ml) = T/R

where *T* is estrogen concentration (pg/ml) obtained from logit-log plot and *R* is the elution efficiency (100% = 1.0).

Gas Chromatography Massspectrometry (GC-MS) Measurement for 17 β-Estradiol

To compare the level of 17 β-estradiol measured by GC-MS, 14 serum samples of 17 β-estradiol were also measured

by HPLC-RIA. The analysis for 17 β-estradiol by GC-MS was performed at LAB GmbH & Co., Neu-Ulm, Germany.

Statistical Analysis

Statistical significance of differences between means was assessed with Student's *t*-test, and *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Measurement of Serum Estrone and Estradiol Extraction and HPLC

The efficiency of the solid-phase extraction technique, determined as recovery of final radioactivity compared with first added tritium-labeled estrogen in serum, was 89.0 and 92.9% for estrone and 17 β-estradiol, respectively. Separation of various steroid hormones in the HPLC system is shown in Figure 1. Estrone and 17 β-estradiol were sufficiently separated from other steroid hormones. After HPLC, the overall elution efficiency for estrone and 17 β-estradiol ranged from 60 to 70%.

Calibration curves for estrone and 17 β-estradiol are shown in Figure 2. The limit of detection of estrone and 17 β-estradiol, defined as mean blank measurement minus 2SD (2 × standard deviation of zero bound) were 1.04 pg/ml and 0.64 pg/ml, respectively. Intra- and interassay coefficients of variation (CV) are summarized in Table 1. The intra-assay CV of estrone and 17 β-estradiol ranged from 19.5 to 28.7%, and 8.5 to 13.7%, respectively. The interassay CV of estrone and 17 β-estradiol was 18.0 and 14.7%, respectively. The spiking recovery of added estrone and 17 β-estradiol was 109.9 and 108.6%, respectively. As shown in Table 1, the overall CV of estrone and 17 β-estradiol in postmenopausal women was 19.5 and 8.5%, respectively. Serum samples were assayed both undiluted and diluted with the zero calibrator. Our procedure maintained good linearities under dilution of estrone and 17 β-estradiol.

GC-MS Measurement of 17 β-Estradiol

Fourteen samples were measured by HPLC-RIA to establish serum levels of 17 β-estradiol and were also analyzed by GC-MS. As shown in Figure 3, the correlation was good (R2 = 0.777).

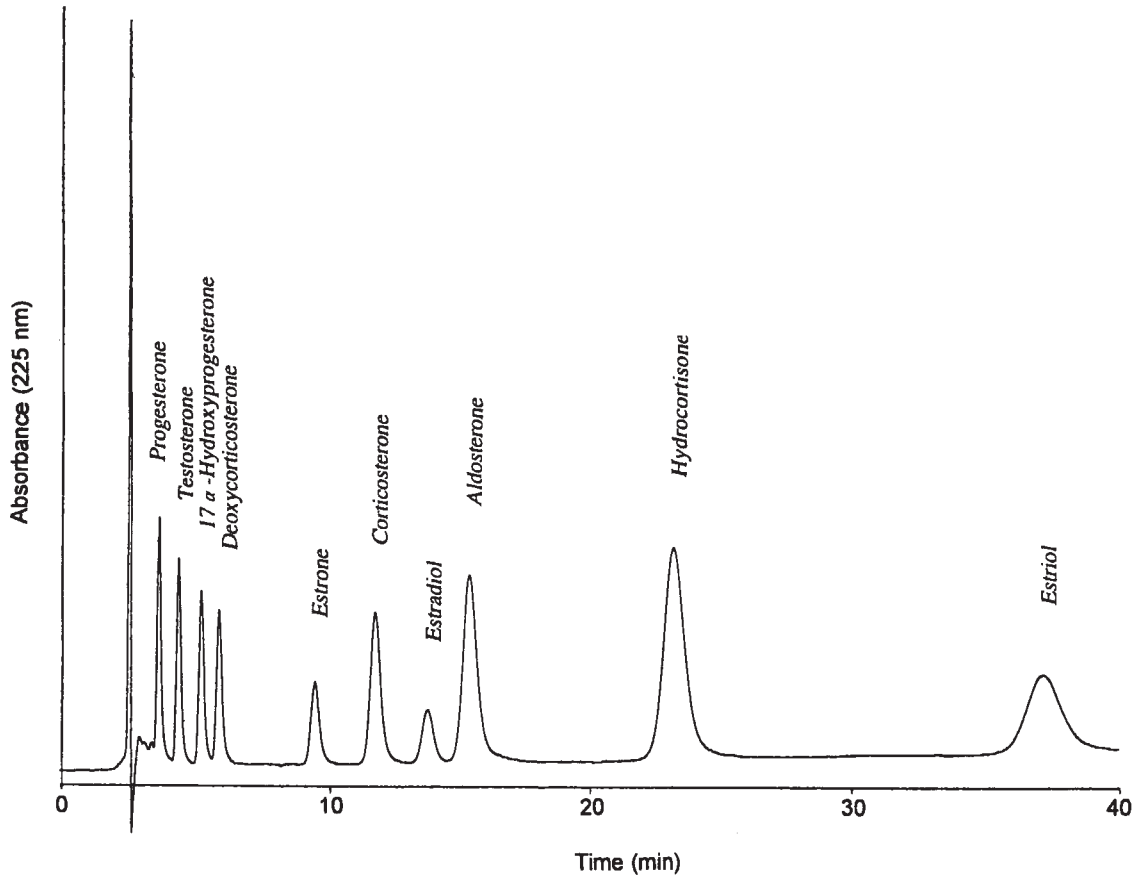

Fig. 1. High-performance liquid chromatogram of a standard mixture of steroids. The HPLC of steroids was performed using CAPCELL PAK NH2 column at a flow rate of 1 ml/min. Elution of steroids was monitored by absorption at 225 nm.

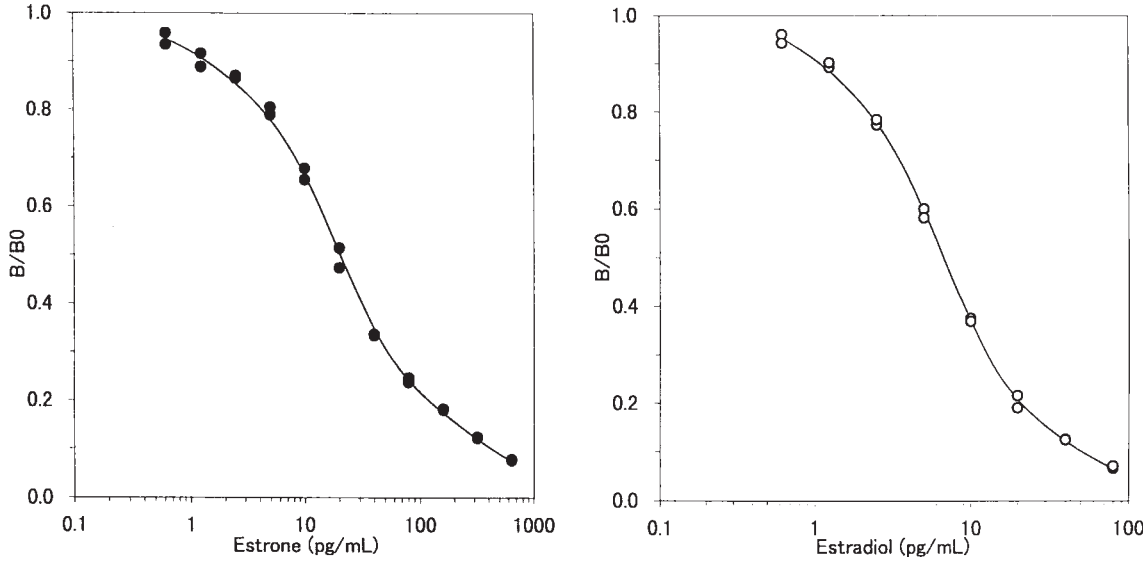

Fig. 2. Calibration curves for estrone (left) and 17 β-estradiol (right) in RIA.

Measurement of Serum Levels of Estrone and 17 β-Estradiol in Perimenopausal Women

The serum levels of estrone and 17 β-estradiol in 154 peri- and postmenopausal women were measured by HPLC-RIA method, and these levels were expressed as the period since their last menstruation (Table 2). The mean level of estrone for the same period was 24.2 pg/ml; this level decreased to 15.7 pg/ml in the postmenopausal period. During a period of 3 to 5 years and 10 to 15 years, these levels increased slightly to 21 and 27 pg/ml, respectively, though no significant differences were found. On the other hand, the mean level of 17 β-estradiol during the period of less than 1 year was 23.6 pg/ml, and thereafter that level decreased to 4 pg/ml. There was little variation in this level up to 10 years after the subject's last menstruation, but it then increased slightly to 6 pg/ml from 10 to 15 years after.

Change in Serum Levels of Estrone and 17 β-Estradiol With a Single Oral Administration of Conjugated Equine Estrone (CEE)

CEE was given orally to 5 volunteer postmenopausal women, and blood samples were taken for the first 3 hr, then at 6, 12, 24, 36 and 48 hs. Figure 4 shows the changes in serum levels of estrone and 17 β-estradiol after oral administration of 0.625 mg CEE. The mean levels of estrone and 17 β-estradiol before administration were 12.2 and 3.0 pg/ml, respectively, and reached a peak of 125.9 and 13.7 pg/ml, respectively, after 12 hr of administration. These levels gradually fell toward the 48-hour

TABLE 1. Reproducibility of estrogen determinations in pooled serum[a]

	Estrone		Estradiol	
Intra-assay	Normal men	Postmenopausal women	Normal men	Postmenopausal women
n	10	7	10	7
Mean (pg/ml)	42.5	22.0	31.5	5.3
SD (pg/ml)	12.21	4.29	4.33	0.45
CV (%)	28.7	19.5	13.7	8.5
Spiking	add 300		add 150	
n	10		10	
Mean (pg/ml)	376		197	
SD (pg/ml)	41.4		17.1	
CV (%)	11.0		8.7	
Yield (%)	109.9		108.6	
Inter-assay				
n	7		7	
Mean (pg/ml)	31.1		33.7	
SD (pg/ml)	5.61		4.97	
CV (%)	18.0		14.7	

[a]Spiking samples were made to add estrogen into normal men serum.

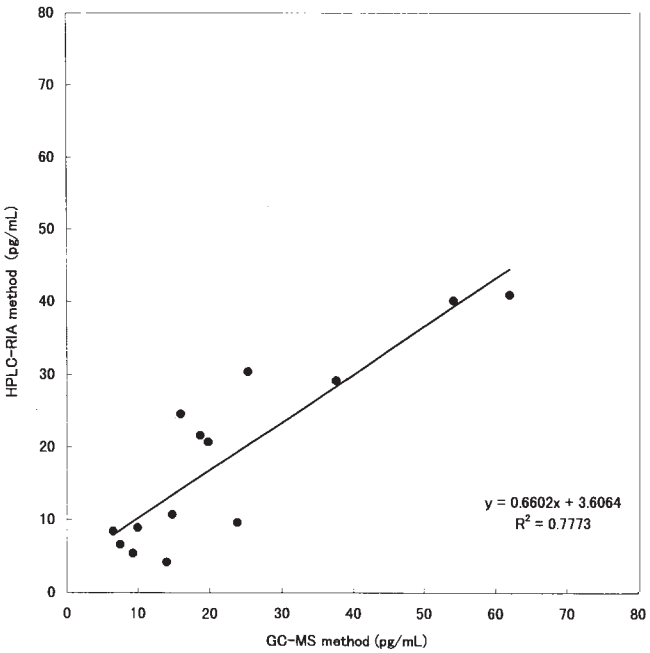

Fig. 3. Correlation of 17 β-estradiol in serum by HPLC-RIA and GC-MS. The correlation was high (R2 = 0.777).

point, when the serum levels of estrone and 17 β-estradiol were 27.2 and 3.6 pg/ml, respectively.

Change in Serum Levels of Estrone and 17 β-Estradiol in Postmenopausal Women Undergoing Hormone Replacement Therapy

Sixty-three postmenopausal women who suffered from vasomotor symptoms or vaginal atrophy were treated with continuous combined hormone replacement therapy consisting of 0.625 mg CEE and 2.5 mg MPA. They were divided into two groups, i.e., everyday and every other day administration. Based on the result of a single administration of CEE, blood samples were drawn before treatment, at 12 to 18 hr after medication, and at 6 months and 12 months in both groups. As shown in Figure 5, the mean level of estrone after 6 months' treatment everyday was significantly increased ($P < 0.0001$) to 131.0 pg/ml, and this level continued to 12 months. On the other hand, the levels of estrone after 6 and 12 months in the group treated every other day were 74.8 and 73.4 pg/ml, respectively. These levels were approximately half

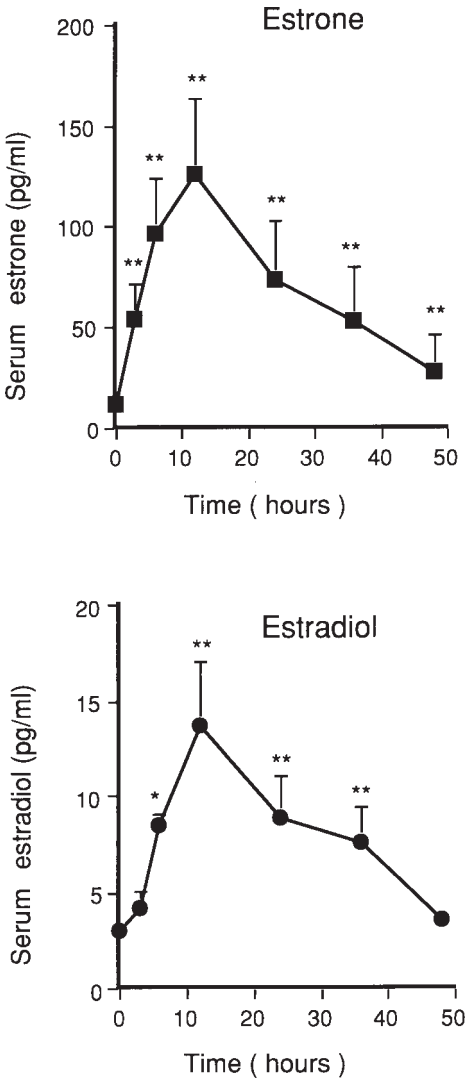

Fig. 4. Estimation of serum levels of estrone and 17 β-estradiol after a single oral administration of 0.625 mg conjugated equine estrogen (CEE). *, $P < 0.01$ vs. before administration; **, $P < 0.05$ vs. before administration. Each value represents the mean ± SE.

those of the group treated everyday. The mean levels of 17 β-estradiol after 6 and 12 months in the group treated every day were 20.3 and 21.3 pg/ml, respectively, whereas those in the group treated every other day were 15.3 and 15.9 pg/ml, respectively.

TABLE 2. Serum levels of estrone and 17 β-estradiol in peri- and postmenopausal women[a]

Period since last menopause (year)	Less than 1	1–2	2–3	3–5	5–7	7–10	10–15	15–20	20–30
No. of women	11	17	21	13	17	27	23	17	8
Estrone (pg/ml)	24.2 ± 11.9	15.7 ± 6.30	16.9 ± 8.60	21.1 ± 21.5	15.0 ± 9.45	17.3 ± 7.97	27.4 ± 29.6	17.3 ± 9.78	22.4 ± 16.7
17 β-estradiol (pg/ml)	23.6 ± 27.6	4.20 ± 2.90	4.50 ± 2.70	4.38 ± 3.58	3.92 ± 2.76	3.54 ± 1.86	6.26 ± 4.19	3.94 ± 1.60	4.33 ± 2.64

[a]Results are mean ± SD.

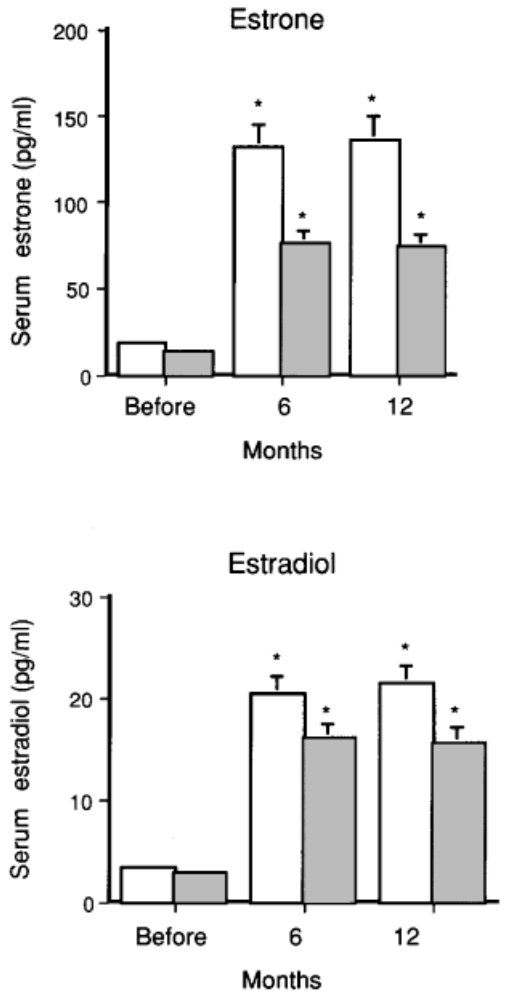

Fig. 5. Mean levels of estrone and 17 β-estradiol in serum of postmenopausal women before and during hormone replacement therapy with conjugated equine estrogen and medroxyprogesterone acetate. Before = before hormone replacement therapy. □, everyday; ▒, every other day; *, $P < 0.01$ vs. before therapy. Each value represents the mean ± SE.

DISCUSSION

We developed a highly sensitive and specific assay of estrone and 17 β-estradiol in serum using the combination of an automatic HPLC-RIA method after extraction. It has been reported that several assay methods use the extraction of steroid hormones from serum (11–14). In these methods, such extractions were performed by liquid–liquid extraction using ether or dichloromethane. We carried out solid-phase extraction using Sep pak tC18 to extract estrogen, as liquid–liquid extraction is complicated and hard to automate. Using a solid-phase extraction robot, it was easy to automate the extraction process.

The method used to separate steroid hormones by HPLC is either gradient elution, that which Schoneshofer and Ueshiba reported using (15,16), or isocratic elution. The gradient elution method is more effective for separating samples that contain many constituents. However, this method requires a high performance gradient controller to obtain reproducibility of retention times. Therefore, we constructed an isocratic elution system using CAPCELL PAK NH2 column. Our HPLC system can effectively separate estrone and 17 β-estradiol from other steroid hormones without special equipment.

A direct RIA method using antiserum must have high specificity to obviate interference with other steroid hormones. However, we selected an antiserum of high affinity rather than of high specificity in order to get higher sensitivity. Consequently, the antiserum against estradiol used in our method showed a 12% cross-reaction with estrone, and therefore the process of HPLC was required in the purification of estrone and 17 β-estradiol. The antiserum of estradiol did not react with the corticosterone and aldosterone that were eluted close to a 17 β-estradiol by HPLC.

We measured the serum levels of estrone and 17 β-estradiol in postmenopausal women using the combination of an automatic HPLC-RIA method after extraction. It was reported that the mean levels of circulating 17 β-estradiol after menopause were approximately 10 to 20 pg/ml (17–19), most of which were derived from peripheral conversion of estrone. On the other hand, the circulating levels of estrone in postmenopausal women ranged from approximately 30 to 70 pg/ml, which were higher than those of 17 β-estradiol. Using our sensitive assay, the mean levels of serum estrone and 17 β-estradiol ranged from 15 to 27, and 3.5 to 6.3 pg/ml, respectively, in postmenopausal women. These levels were lower than those reported previously.

Postmenopausal women had very low levels of estradiol, which is associated with loss of bone mineral density, vasomotor symptoms, atrophy of reproductive organs, and cardiovascular disease. Hormone replacement therapy can relieve vasomotor symptoms, increase bone mineral density, and decrease the circulating level of total cholesterol (20), and can therefore prevent osteoporosis and cardiovascular disease. Chetkowski reported that tissue sensitivity to estradiol varies, and that hierarchy in the estrogen-responsive process, from the most sensitive to the least, is calcium turnover, gonadotropin secretion, vaginal epithelial growth, lipid production, and liver protein production (9). Breast cancer is very sensitive to the growth-promoting effects of estradiol as low as 10 to 20 pg/ml. Therefore, it is necessary for postmenopausal women receiving hormone replacement therapy to monitor the serum levels of 17 β-estradiol. However, there are some difficulties in clinical assays as techniques and quality differ, and CEE used widely for hormonal therapy represents a diverse collection of estrogenic compounds, ranging from estradiol to unique equine estrogens. Using our HPLC-RIA method, we could accurately monitor the levels of estrone and 17 β-estradiol in postmenopausal women receiving CEE.

We first measured the serum levels of estrone and 17 β-

estradiol with a single administration of CEE. The serum levels of estrone and 17 β-estradiol similarly increased, reached a peak at 12 hr after administration, and then gradually decreased. Based on these results, we measured the serum level of estrone and 17 β-estradiol at 12 to 18 hr after receiving daily combined administration of CEE and MPA in postmenopausal women who suffered from vasomotor symptoms. The problem of greatest concern in daily combined hormone replacement therapy is breakthrough bleeding (21). Therefore, we have used combined hormone therapy every other day, which relieves vasomotor symptoms and decreases the incidence of genital bleeding in Japanese women (22). This regimen is effective in postmenopausal women wishing to eliminate genital bleeding and reduce both hormonal side effects and menopausal symptoms. The mean level of estrone at 12 months after daily combined hormone replacement therapy was 135.0 pg/ml, a level which was approximately twice the level of the group treated every other day. On the other hand, the mean level of 17 β-estradiol at 12 months in the group treated everyday was 21.3 pg/ml, which was slightly higher than the level in the group treated every other day. The increase in estrone was higher than that of estradiol.

Recently, Ettinger and coworkers found a protective effect of low levels of estradiol against low bone mass and fracture. Those who had estradiol levels from 10 to 25 pg/ml had a greater level of bone mineral than did those with levels below 5 pg/ml (23). Our method allows one to determine very low levels of estrone and 17 β-estradiol in postmenopausal women and shows accurate levels of such hormones during hormone replacement treatment. This method may be clinically very useful not only for evaluating ovarian function but also for monitoring treatment with CEE as hormone replacement therapy in postmenopausal women.

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