Research Paper

Development and validation of the simultaneous measurement of estrone and 17-β estradiol in serum by LC-MS/MS for clinical laboratory applications

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Abstract Application of liquid chromatography tandem mass spectrometry (LC-MS/MS) in clinical chemistry has been increasing worldwide, especially in large institution and reference laboratories. Although immunoassays are often used for measurement of serum estradiol (E2) when fast turnaround time is required, more sensitive and specific measurements are needed for determination of menopausal status, estrogen deficiency and in the diagnosis of sex hormone related disorders. Furthermore, simultaneous measurement of estrone (E1) and E2 is often requested particularly from gynecologic oncologists. Indeed, increased risk of endometrial cancers has been shown in subjects with high serum estrogen levels. The aim of this study is to develop and validate LC-MS/MS method for simultaneous measurement of E1 and E2 in human serum. Serum samples were first prepared in a 96-well supported liquid extraction plate and the eluate was derivatized by the dansyl chloride acetone solution. The derivatized samples were subjected to LC-MS/MS, and detected by selected reaction monitoring. The lower limits of quantification for E1 and E2 were 6.2 and 7.3 pg/mL, respectively. The 60 female sera values obtained by the LC-MS/MS method were compared with those obtained by two commercially available immunoassays. The both of values obtained by the two immunoassays exhibited positive bias particularly at low E2 levels. Various pre-analytical factors, such as long sample sitting prior to serum separation, repeated freeze−thaw cycles, and the presence of anticoagulants, had no significant effects on these determinations. This method will aid further understanding of low-abundance estrogen, as well as the accurate determination of E1 and E2.

Key words: estrone, 17-β estradiol, dansyl chloride

Abbreviations: E1, estrone; E2, 17-β estradiol; QC, quality control; IS, internal standard; ESI, electrospray ionization; LLOQ, lower limit of quantification; CV, coefficient of variation

Introduction

Estradiol is the most potent estrogen and plays the major roles in the development and maintenance of female sex organs and also in regulation of the menstrual cycle. Since the first radioimmunoassay (RIA) was reported in 1969, serum estradiol levels have been mostly measured by immunoassays for decades[1)]. In recent years, RIAs are increasingly replaced by non-RIA assays such as chemiluminescent immunoassay (CLIA) mainly because handling radioactive materials is a kind of nuisance hospital-based

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clinical laboratories[1]. For the purpose of evaluation of ovarian function in which demands on assay sensitivity is modest, immunoassays can provide relevant results. However, there is increasing clinical demands on highly sensitive estradiol assays for the diagnosis of variety of disorders such as inborn errors of sex steroid metabolism and precocious puberty[1]. Highly sensitive assays are also required for quantitation in men, post-menopausal women and in subjects on anti-estrogen treatment[1]. Current immunoassays are not sensitive and accurate enough for estradiol measurements under these circumstances. Indeed, it was reported in a study comparing the analytical performances of the eight commercial estradiol immunoassays that their coefficient variation values ranged from 6.9 to 42.6% at 18 pg/mL level[2].

The most accurate method for determination of serum estradiol level is gas-chromatography mass spectrometry (GC-MS)[3]. But throughput of GC-MS is not satisfactory for routine clinical use. In terms of sensitivity and throughput, liquid chromatography with tandem mass spectrometry (LC/MS/MS) is increasingly used[4–7]. The advantages of the use of LC-MS/MS for steroid hormone measurement over immunoassays are well recognized by one of the distinguished journal in endocrinology, the Journal of Clinical Endocrinology and Metabolism. A position statement released from the journal in 2013 indicated that "effective January 1, 2015, manuscripts reporting sex steroid assays as important endpoint must use MS-based assays"[8]. Although this is a controversial subject and the journal policy on this issue has been suspended for various reasons, the position statement will definitely act as an impetus toward accelerating use of LC-MS/MS in sex steroid measurements.

Although only one analyte can be measured per one immunoassay, LC/MS/MS permits simultaneous determinations of multiple analytes by one run. Estrone, another key estrogen, is a major form in post-menopausal females and simultaneous measurement of estradiol and estrone is significant not only for better understanding of female physiology, but in the field of gynecological oncology. Circulating estrogens contribute to development of endometrial cancer and ovarian cancer[9, 10]. Since increased serum estrone levels are reported to be positively associated with ovarian[11] and endometrial[12] cancer risk, requests for simultaneous measurements of estradiol and estrone are going to be increased.

The aim of this study is to develop and validate an LC/MS/MS method to simultaneously measure estradiol and estrone in serum for application in clinical laboratories. Also, we determined the effects of various preanalytical factors such as interferences, sample collection processes, and the presence of anticoagulants on these determinations.

Materials and Methods

Calibrators and quality controls

Mass spectrometry-grade solvents and formic acid were from Wako Pure Chemical Industries (Osaka, Japan). Water was from Nacalai Tesque (Kyoto, Japan). Estrone, 17-β estradiol and 17-β estradiol-$^{13}C_3$ were purchased from Sigma-Aldrich (St Louis, MO, USA). Estrone-$^{13}C_3$was purchased from IsoSciences (King of Prussia, PA, USA). Estrone-$^{13}C_3$ and 17-β estradiol-$^{13}C_3$ were used as internal standards (ISs). The IS working solution was prepared in 50% methanol and contained estrone and 17-β estradiol at each concentration of 10 ng/mL. The calibrators of Estrone and 17-β estradiol were purchased from Biocrates Life Sciences AG (Innsbruck, Austria). The concentrations of the 4 Biocrates calibration standards for estrone and 17-β estradiol were as follows: 30 and 20 pg/mL for calibrator level 1; 120 and 80 pg/mL for calibrator level 2; 600 and 400 pg/mL for calibrator level 3; and 1200 and 1600 pg/mL for calibrator level 4. Human Serum Standard Reference Material (BCR576, BCR577 and BCR578) as the quality control (QC) sample was from Sigma-Aldrich. The quality control value for 17-β estradiol in BCR576, BCR577 and BCR578 was 31.5±1.36, 187.95±10.88 and 365.00±19.04 pg/mL, respectively.

Human serum samples and handling procedures

A total of 60 consecutive sera obtained from blood samples requested for estradiol measurements in Clinical laboratory of Chiba University Hospital were used to assess correlation between LC-MS/MS and chemiluminescent immunoassay (CLIA) measurements. The study protocol (No. 685) was approved the ethics committee of our institute and each subject provided written informed consent prior to their enrollment in the study. Three serum samples were obtained from 3 apparently healthy volunteers with various levels of serum E1 and E2 concentrations to validate the precision of this method. Venous blood samples were collected in vacutainer tubes (4S1001, Insepack. II, Sekisui Medical Co., Ltd., Tokyo, Japan) which contained SiO_2 as a coagulation enhancer and the samples were left at

room temperature for 30 min. The sera were then obtained by centrifugation at 1200 × *g* for 10 min at 4°C. The ethics committee of our institute approved the study protocol, and each subject provided written informed consent prior to their enrollment in the study.

Sample preparation and Dansyl chloride derivatization

The serum samples (100 μL) were diluted by equal volume of water. IS solution (20 μL) was added to diluted serum and vortexed for one minute. Samples were applied individually to the wells of a 96 well supported liquid extraction (SLE) plate (ISOLUTE SLE+: Biotage, Uppsala, Sweden) and incubated for five minutes. The analytes were eluted three times with 600 μL ethyl acetate : hexane (75 : 25, v/v), then the samples were evaporated to dryness under nitrogen at 45°C using a Biotage SPE Dry 96 (Biotage). The dry samples were dissolved in 50 μL of sodium bicarbonate buffer (100 mM, pH 10.5) and 50 μL of dansyl chloride solution (1.0 mg/mL in acetone), then vortexed for 1 min. The mixed samples were incubated at 60°C for 10 min, then cooled at 4°C and a 40 μL of aliquot was injected onto the LC-MS/MS.

LC-MS/MS conditions

The reconstituted samples were analyzed by Advance HPLC and EVOQ Elite triple quadrupole mass spectrometer (Bruker Daltonics Inc., Fremont, CA, USA) using the following conditions. The estrone and 17-β estradiol were separated on a Shiseido core shell column (CAPCELL CORE C18, 2.1 × 150 mm) maintained at 40°C. The HPLC flow rate was 500 μL /min and the solvent composition of the mobile phase was programmed to change in 10-min cycles with varying mixing ratios of solvent A (H_2O containing 0.1% v/v HCOOH) to solvent B (CH_3CN containing 0.1% v/v HCOOH): 50−70% B 1 min, 70−80% B 7 min, and 50% B 2 min. Samples were ionized using an electrospray ionization (ESI) source using the following conditions: positive-ion mode; spray voltage, 5000 V; heated probe temperature, 450°C; cone temperature, 300°C. Selected reaction monitoring (SRM) transitions of analytes were monitored: 504 → 171 for estrone, 507 → 171 for estrone-$^{13}C_3$, 506 → 171 for 17-β estradiol, 509 → 171 for 17-β estradiol-$^{13}C_3$. The collision energy of each analytes was set at 38 V for estrone, 34 V for estrone-$^{13}C_3$, 34 V for 17-β estradiol, 33 V for 17-β estradiol-$^{13}C_3$. The scan time was set at 50 msec for all analytes.

LC/MS/MS assay validation

Accuracy was estimated by measuring twenty replicates of 17-β estradiol in BCR QC sera. Control sera of low, medium and high level estrone and 17-β estradiol concentrations were used for assessing assay precision. On 20 consecutive days, estrone and 17-β estradiol were measured with four replicates per day (inter-day) of three level control sera. On the day different from the 20 consecutive days, estrone and 17-β estradiol were measured with 20 replicates of the three level control sera per day (intra-day). Dilution linearity was evaluated by measuring duplicates of five dilutions of the two concentration levels of the control sera (medium and high) for estrone and the QC serum (BCR578 and BCR576 : BCR577=8 : 2 (v/v)) for 17-β estradiol. The lower limit of quantification (LLOQ) was determined from four replicate measurements of high level control serum for estrone and BCR578 for 17-β estradiol diluted with saline as follows: 1 : 1 (not diluted), 1 : 2, 1 : 5, 1 : 10, 1 : 20, 1 : 50 and 1 : 100. The LLOQs were set to the level at which dilution linearity was confirmed, S/N ratio was＞10 and the coefficient of variance was ＜10%.

Evaluation of preanalytical factors of estrone and 17-β estradiol measurements

Effects of various preanalytical factors on the simultaneous measurement of estrone and 17-β estradiol were assessed. Except for the experiments to test the effects of various clotting time, serum samples were obtained by centrifugation 30 min after venipuncture. Except for the effects of anti-coagulants or separating gel, serum samples were collected by Insepack. II serum collection tubes (4S1001, separating gel and silica, accelerating clotting agent used as the additive).

Stability: To test stability of estrone and 17-β estradiol, serum samples obtained from four healthy volunteers were left at 4°C for 1, 6, 24, 72 and 168 h prior to measurement.

Freeze and thaw: To test the effects of freeze-thaw cycles, serum samples were frozen at −80°C in a deep freezer and then thawed at 37°C. The samples underwent 1 to 5 freeze-thaw cycles.

Effects of anti-coagulants and separating gels: To determine the effects of various anti-coagulants (EDTA, heparin, sodium citrate), separating gels and accelerating clotting agent (SiO_2) on our LC-MS/MS based measurement of estrone and 17-β estradiol, a total of six different blood collection tubes were used; (I) plasma tubes (4H2011, Sekisui

Medical, sodium heparin (15 IU/mL)), (II) plasma tubes (4E4090, Sekisui Medical, dipotassium EDTA (1.5 mg/mL)), (III) plasma tubes (4E2034, Sekisui Medical, disodium EDTA (1.5 mg/mL)), (IV) serum tubes (4S1001, Sekisui Medical, separating gel, silica and SiO_2), (V) serum plain tubes (VP-P070K, Terumo, no additive) and (VI) serum tubes (VP-AS076K, Terumo, separating gel, glass particles and SiO_2).

Interferences: Potential interferences were tested by spiking to high level control serum with free bilirubin, conjugated bilirubin, chyle and hemolyzed hemoglobin by using the Interference Check A Plus (Sysmex Co., Hyogo, Japan) including (maximum final concentration) 20.1 mg/dL of free bilirubin, 20.9 mg/dL of conjugated bilirubin, 1480 formazin turbidity unit (FTU) for chyle and 520 mg/dL of hemoglobin.

Chemiluminescent immunoassay (CLIA) measurements for 17-β estradiol

The 17-β estradiol concentration of 60 female sera were determined by the two representative commercial chemiluminescent immunoassay (CLIA), ARCHITECT Estradiol II kit (Abbott, Chiba, Japan) and ADVIA Centaur Chemilumi E-estradiol-6 kit (Siemens, Tokyo, Japan) according to the manufacturers' instructions.

Results

Evaluation of the LC-MS/MS assays

The SRM chromatograms of estrone and 17-β estradiol standards are shown in Fig. 1. The HPLC method provided adequate separation of the estrogen, with 17-β estradiol eluting at 5.36 min and estrone eluting at 6.20 min. Each internal standard was eluted at the same time as the standard reference material. In addition, 17α-estradiol was eluted at 5.36 min and each transition did not interfere with each other. The accuracy values of 17-β estradiol measurement were 100.7−100.9% (Table 1). The coefficient of variation (CV) for the 17-β estradiol was lower than 4.5% at all QC levels. The precision of this assay was determined by using control sera containing low (21.3±1.39 and 12.5±1.13 pg/mL), medium (52.4±2.68 and 33.2±2.10 pg/mL) and high (124.6±2.33 and 223.0±3.03 pg/mL) level estrone and 17-β estradiol concentrations, respectively. The intra-assay CVs of estrone and 17-β estradiol measurements were 5.3% and 7.2% for the low level samples (low), 2.7% and 6.4% for the medium level samples (medium) and 1.7% and 0.96% for the high level samples (high), respectively. The inter-assay CVs for estrone and 17-β estradiol were 2.0% and 3.1% for the low, 5.5% and 5.1% for the medium and 1.5% and 1.1% for the high level samples, respectively (Table 2). The results of the dilution linearity tests for the estrone and 17-β estradiol are shown in Fig. 2a. The correlation coefficients at the all levels were more than 0.9993 and 0.9968 for estrone and 17-β estradiol, respectively. The LLOQ were determined by four replicate measurements of the medium level serum and BCR578 dilution for estrone and 17-β estradiol, respectively. The LLOQs were 6.2 and 7.3 pg/mL for estrone and 17-β estradiol, respectively (Fig. 2(b)).

Effects of various preanalytical factors on measurement of estrone and 17-β estradiol by LC-MS/MS

In the clinical laboratory, it is a common practice to save the rest of the clinical samples just in case for possible re-testing. The estrone and 17-β estradiol were found to be stable when left as serum samples up to 7 days at 4°C (Fig. 3(a)).

Our results showed that repeated freeze-thaw cycles for up to 5 times had no significant effect on the estrone and 17-β estradiol serum concentrations, the CVs of the concentration of estrone and 17-β estradiol for the 4 data points were 1.9−4.7% and 1.8−3.3%, respectively (Fig. 3(b)).

Previous study has shown that the gel in blood collection tubes causes interference in one of the main testosterone transitions[13]. We investigated the effects of anti-coagulants and separating gel on the estrone and 17-β estradiol assay by examining a total of six different blood collection tubes (Fig. 3(c)).

No significant differences were noted in the estrone and 17-β estradiol concentrations for any of the blood collection tubes. The potential interferences by hyperbilirubinemia, hemolysis and cylemia were tested using commercially available interference test kits. No significant effects were noted as indicated in Fig. 3(d).

Comparison of serum 17-β estradiol levels measured by the in-house LC-MS/MS method and by two commercial CLIA kits

Serum levels of E2 and E1 in 60 female subjects determined by the LC-MS/MS method were compared with those by the commercially available Abbott CLIA and Siemens CLIA.

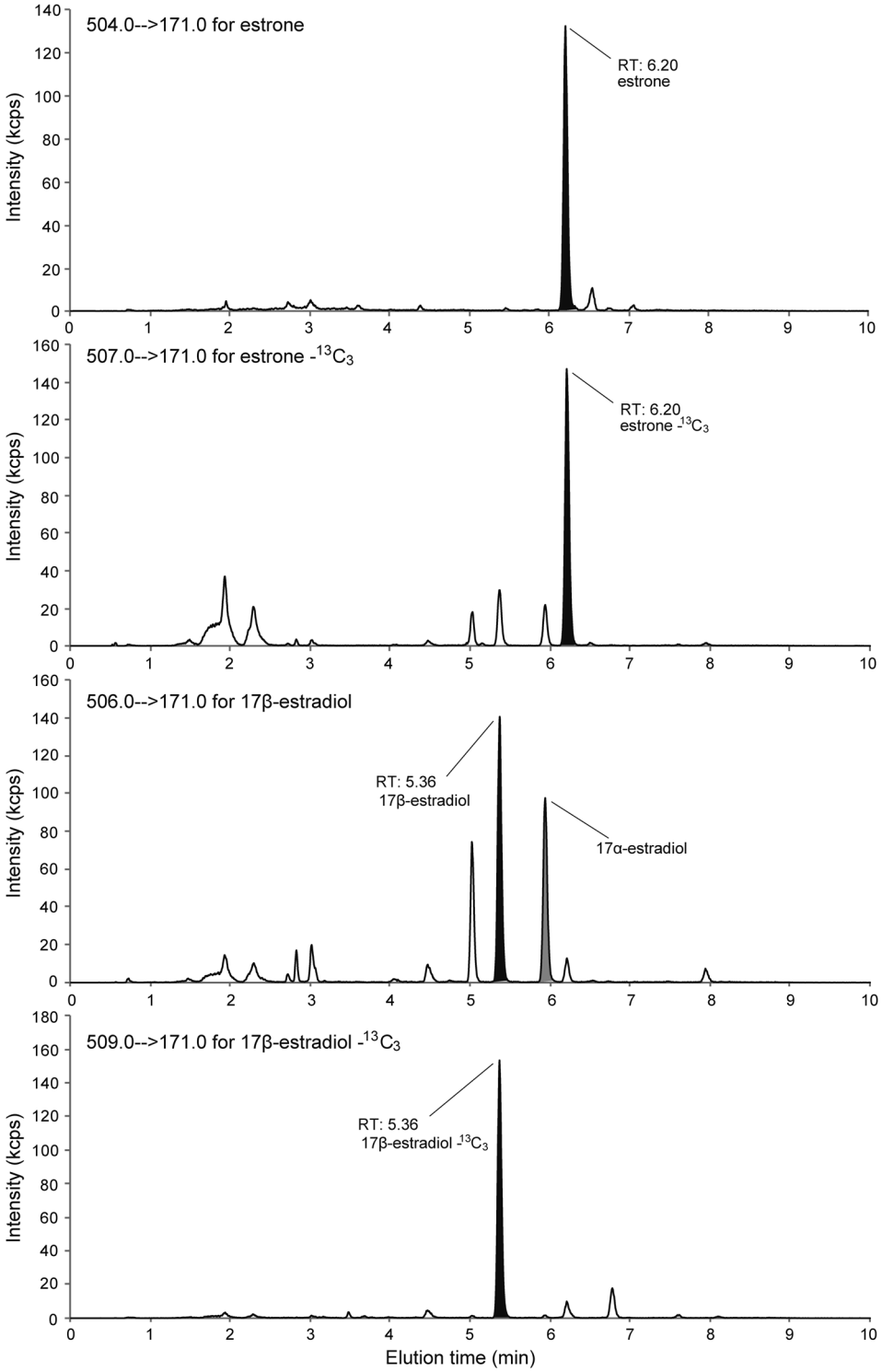

Fig. 1. SRM chromatograms of estrone and 17-*β* estradiol and the ISs.

From the top panel to the bottom panel shows 504→171 for estrone, 507→171 for estrone-$^{13}C_3$, 506→171 for 17-*β* estradiol and 509→171 for 17-*β* estradiol-$^{13}C_3$. The black peak is the target analyte peak. The gray peak is 17-*α* estradiol and can be separated from all peaks (estrone, 17-*β* estradiol and ISs).

The ranges of the concentrations in 60 sera were 11−872, 18−959 and 7.6−948.7 pg/mL as measured by the Abbott CLIA kit, the Siemens CLIA kit and LC-MS/MS, respectively. The linear regression line and correlation coefficient were y (Siemens)=1.1052x (Abbott)−0.0288 and 0.9880, respectively. When the values obtained by the LC-MS/MS (x-axis) were compared with the values obtained by two CLIA kits (y-axis), good correlations were obtained, but

y-intercepts were large (Fig. 4(a), (b)). In comparison with the Abbott kit, the linear regression line and correlation coefficient were *y* (Abbott)=0.8597*x* (MS)+15.652 and 0.9951, respectively. In comparison with the Siemens kit, the linear regression line and correlation coefficient were *y* (Siemens)=0.959*x* (MS)+15.258 and 0.9924, respectively. Surprisingly, the biggest discrepancy between the LC-MS/MS method and the two CLIA methods were seen in the same specimen for which the LC-MS/MS gave 11.9 pg/mL: the values obtained by the Abbott CLIA and the Siemens CLIA were 34 and 48 pg/mL, respectively.

Table 1. Accuracy of the LC-MS/MS assay for 17-*β* estradiol

Standard	Expected conc. (pg/mL)	Average (pg/mL)	SD. (pg/mL)	CV. (%)	Accuracy (%)
BCR576	31.5±1.36	30.84	1.08	4.5	100.7
BCR577	187.95±10.88	186.99	2.43	1.6	100.9
BCR578	365.00±19.04	367.56	2.56	1.3	100.8

Table 2. Intra-and Inter-day precisions of the LC-MS/MS assay for estrone and 17-*β* estradiol

Compound		Conc. (pg/mL)	Intra-day CV (%)	Inter-day CV (%)
Estrone	Low	21.3±1.39	5.3	2.0
	Medium	52.4±2.68	2.7	5.5
	High	124.6±2.33	1.7	1.5
17-*β* estradiol	Low	12.5±1.13	7.2	3.1
	Medium	33.2±2.10	6.4	5.1
	High	223.0±3.03	0.96	1.1

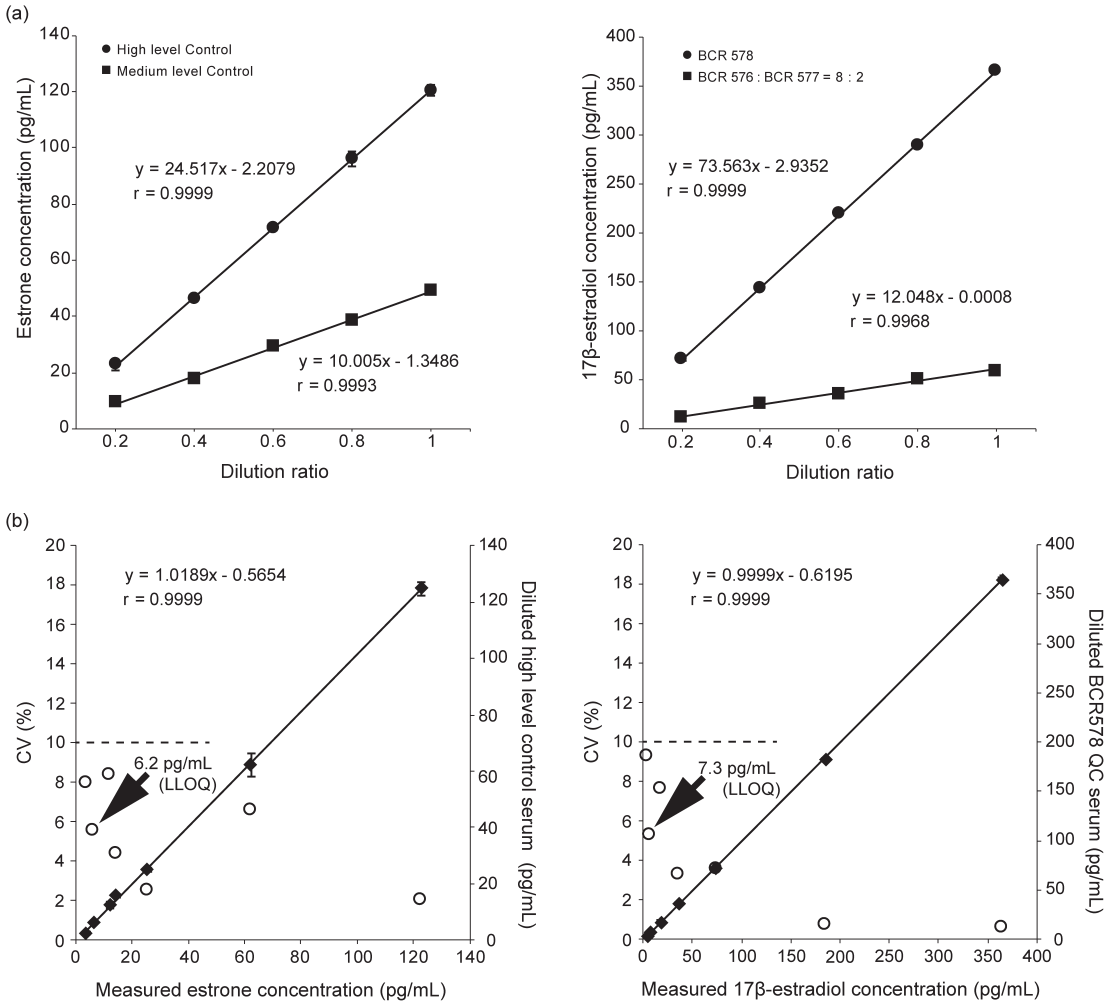

Fig. 2. Dilution linearity and LLOQ.

(a) Dilution linearity of estrone and 17-*β* estradiol. Dilution linearity was evaluated by measuring duplicates of 5 dilutions of each levels serum. (b) LLOQ of estrone and 17-*β* estradiol measurements. Diamonds and open circles indicate values obtained by BCR578 dilution series and %CV at each dilution concentration, respectively. Arrowheads indicate LLOQ that meets our criteria.

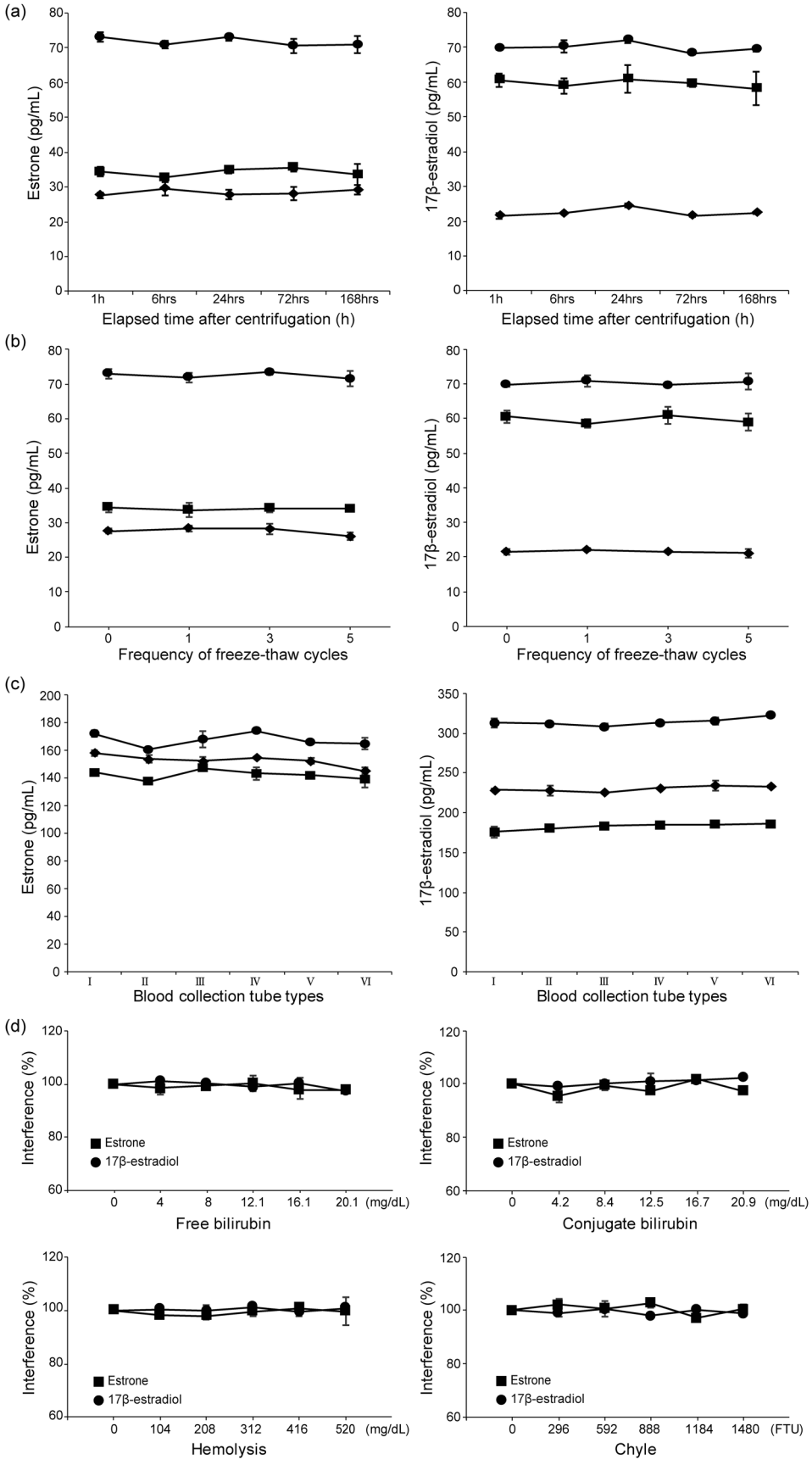

Fig. 3. Effects of various preanalytical factors.

(a) Stability. (b) Freeze and thaw. (c) Effects of anti-coagulants and separating gels. (d) Interferences.

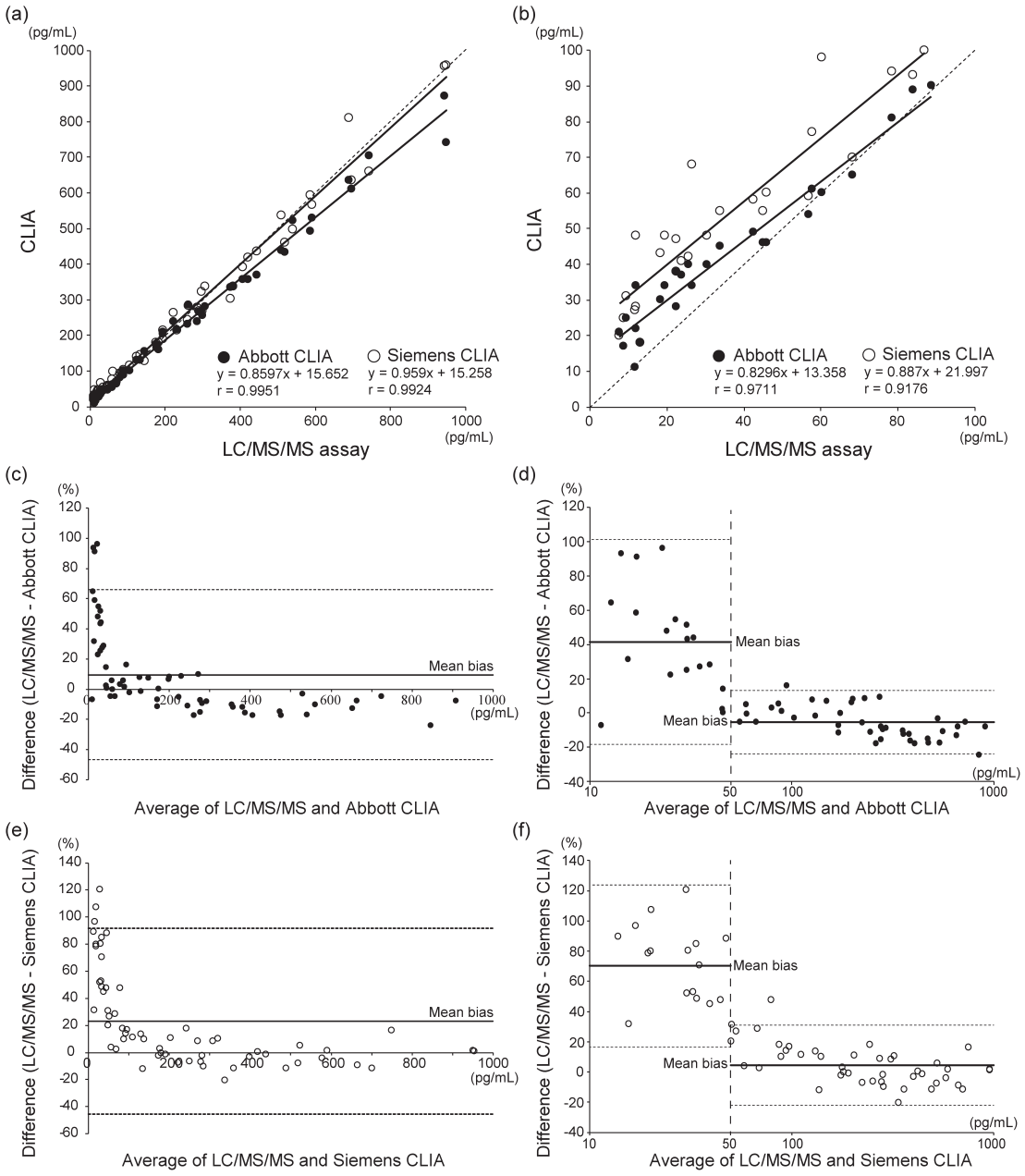

Fig. 4. Linear regression analysis of the two commercial CLIA kits and in-house LC/MS/MS measurements of 17-*β* estradiol. (a) The correlation between 17-*β* estradiol measured by CLIA and by LC/MS/MS. (b) Expanded view of low concentration in (a). (c and d) Bland−Altman plot of measurement value by Abbott CLIA kit and LC-MS/MS. (e and f) Bland−Altman plot of measurement value by Siemens CLIA kit and LC-MS/MS. (d and f) Recalculated the bias with 50 pg/mL as the boundary.

Discussion

Here we described a simple, rapid and reproducible LC-MS/MS method for the simultaneous assays of estrone and 17-*β* estradiol considering applications into clinical laboratory. We have adopted supported liquid extraction (SLE) for sample preparation and dansyl chloride as a derivatization reagent.

Protein precipitation (PP), liquid−liquid extraction (LLE), solid phase extraction (SPE), or combinations of these methods, are generally used for sample preparation in LC-MS/MS-based estrogen measurements. Although PP and LLE are simple and not costly, phospholipid, a representative cause of matrix effects, cannot be sufficiently removed by PP[14], and automated preparation of microscale specimen is difficult for PP and LLE because liquid phase separation is needed. For the purpose of practical use of LC-MS/MS, sample preparation requires to be fully automated eventually, as is the case with currently available autoanalyzers in clinical chemistry laboratories. SPE is one candidate for automated preparation because various relat-

ed-products such as single cartridge and 96-well plate are available. There is a limitation, however, because a lot of processes such as washing column, equilibrating and washing after sample loading are necessary, which in turn will lead to low throughput. To overcome these problems, several sample preparation methods other than the three basic methods have been reported. Pucci et al. reported a method using HybridSPE, in which PP and zirconia-coated silica are packed for SPE to remove phospholipids[15]. Geib et al. reported a method using AC Extraction Plate, which was used as an immobilized sorptive material[16]. Supported liquid extraction (SLE) which we have used in Vitamin D measurements[17] is also another promising sample preparation method. With SLE, we can perform preparation by using diatomaceous earth as filler, and interferences can be reduced by efficiently removing phospholipids when the extraction conditions were carefully optimized[18]. In this study, we decided to use SLE because it may have the greatest potential for automation of sample preparation because number of processing steps is small, execution time is short and also because amount of waste is small.

Derivatization is still a key technology to improve ionization efficiency and sensitivity by ESI[17]. The 17-β estradiol is a well-known analyte that has low ionization efficiency at ESI. For this reason, measurement of 17-β estradiol is generally derivatized mostly by dansyl chloride[4]. Recently, 17-β estradiol measurement method without derivatization has been reported using high-end model instruments[5–7]. However, the sensitivities reported in these reports are comparable to those obtained by the derivatization methods. Since high-end models are expensive and require highly-trained staff to handle, they are not suitable for routine use in clinical laboratories of standard-scale hospitals. Therefore, in the present study we decided to use MS equipment that has been claimed to be suitable for use in clinical laboratory.

The LLOQ obtained for E2 in the present study was 7.3 pg/mL and was comparable to those reported in the previous reports[4–7]. But the method we describe here is still not sensitive enough for some clinical situations such as determination in patients under aromatase inhibitor therapy. Aromatase inhibitors inhibit aromatase mediated conversion of androgens, resulting in suppression of estrogen biosynthesis. The third generation aromatase inhibitors such as exemestane and letrozole are effective for the treatment of estrogen-receptor positive breast cancer[19]. LLOQ as low as 0.1−0.2 pg/mL is demanded to assess whether estrogen suppression is enough or not. For this purpose, highly sensitive method such as ultrasensitive gas chromatography tandem mass spectrometry has been used[20]. Although LLOQ was equivalent to the previous report, 200 to 500 μL of serum samples had been used in the previous reports[7,21]. The method we described here requires only 100 μL of serum, which is an advantage for pediatric use in which sample volumes are limited.

Since our goal is to apply this method to real-world clinical laboratories, we determined the effects of various preanalytical factors on the LC/MS/MS measurements of serum levels of E1 and E2. Indeed it was reported that analysis of sera from blood collected in tubes containing clotting activators gave results of testosterone that were fourfold higher than that collected in plain tubes[13]. In the present study, E1 and E2 levels in samples obtained in a total of six different blood collection tubes were comparable.

Serum E2 levels obtained by our LC/MS/MS method were compared with those by the two representative immunoassays available in Japan. Positive bias was noted for both immunoassays at low concentrations less than 50 pg/mL. Similar bias was reported in the previous comparative studies of immunoassays and GC-MS or LC-MS[22,23]. Cross reactivity due to the limit of antibody specificity and matrix effects may be possible reasons for the discordant results[24]. Importantly, accuracies of the calibrators included in each immunoassay may have to be carefully evaluated as well.

Under situations in which demands on assay sensitivity is modest as for the purpose of evaluation of ovarian function, the conventional immunoassays can provide relevant results. It is likely, however, that immunoassays are going to be replaced by more specific and accurate LC/MS/MS for sex steroid measurements as proposed in the editorial of a representative journal in endocrinology[5]. Antibodies may also be useful for the purpose of immunoaffinity extraction in LC/MS/MS based measurements of steroid as we recently reported for 1,25-deihydroxyvitamin D quantification[25]. This immunoaffinity extraction resulted in less background noise and less phospholipid effects compared to the conventional pretreatments[25].

Thus, we developed a simple and reproducible LC/MS/MS method for determination of serum levels of E1 and E2 in clinical laboratories with sensitivities that can meet the majority of clinical needs. Request of simultaneous measurements of E1 and E2 are going to be increased in the field of gynecological malignancy. Our LC-MS/MS method

may be appropriate for this purpose. Worldwide, increasing number of laboratories is measuring E1 and E2 by their home-brew LC/MS/MS methods. Significant interlaboratory variability has been recognized even among LC-MS E2 assay[26]. Harmonization of the different LC/MS/MS methods will be needed to make MS-based steroid assays more reliable and successful.

Acknowledgments

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Conflict of Interest

All authors declare that they have no conflict of interest.

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