High-Sensitivity Tandem Mass Spectrometry Assay for Serum Estrone and Estradiol

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Abstract

High-sensitivity measurement of serum estrogens is important in adult and pediatric endocrinology and oncology. We developed a high-sensitivity liquid chromatography–tandem mass spectrometry (LC-MS/MS) assay for simultaneous measurement of estrone (E_1) and estradiol (E_2). Aliquots of 200 µL of serum were spiked with internal standard, extracted, derivatized with dansyl chloride, and analyzed by LC-MS/MS using 2-dimensional chromatographic separation. Total imprecision for the method was less than 11%; the limit of quantitation was 1 pg/mL. Reference intervals were established with samples from more than 900 healthy postmenopausal women, men, girls, and boys. Concentrations of estrogens in children reached adult levels by Tanner stage 3. In men and postmenopausal women, the median concentrations of total estrogens ($E_1 + E_2$) were 39 and 22 pg/mL, and the median E_2/E_1 ratios were 0.98 and 0.55, respectively. The method requires a small sample volume and has adequate sensitivity and specificity for analyzing estrogens in samples from postmenopausal women, men, and children.

Estrogens are hormones that are responsible for the development and maintenance of female secondary sex characteristics, reproductive function, regulation of the menstrual cycle, and maintenance of pregnancy. High-sensitivity methods for accurate measurement of estrogen concentrations are necessary for the diagnosis of sex hormone–related disorders, oligomenorrhea and amenorrhea, menopausal status, precocious puberty, estrogen deficiency, and antiestrogen treatment.[1-7] Studies also suggest that low concentrations of estrogens in both sexes correlate with osteoporosis and cardiovascular and neurologic diseases.[8-13]

Estrogens have their highest biologic activity in the 17β-hydroxy configuration. Reductive 17-hydroxysteroid dehydrogenase (17HSD) activity is essential for the biosynthesis of the sex hormones. Eleven 17HSD isoenzymes have been identified that differ in tissue distribution, specificity, subcellular localization, and mechanism of regulation.[14] Three enzymes, 17HSD types 1, 3, and 7, participate in the interconversion of 17β-hydroxysteroids and 17-ketosteroids in the gonads, thereby regulating biologic activity.[14-16] The activity of 17HSD is not limited to steroidogenic tissues, and, in addition to circulating hormone, local interconversion of sex steroids by 17HSDs modulates tissue-specific biologic activity.[14-16]

Challenges in measurement of estrogens in the blood of postmenopausal women, men, and children are related to low physiologic concentrations and the presence of endogenous compounds that can interfere. Analysis of estrogens in biologic samples is commonly performed using immunoassays.[17,18] Mass spectrometry–based methods are generally preferred for measurement of steroids because of better specificity.[19-21] Lee et al[21] compared 3 indirect and 4

direct assays for measurement of 17β estradiol (E_2) with a gas chromatography–tandem mass spectrometry (MS/MS) assay and confirmed a lack of agreement between E_2 assays at low concentrations. Mean measured concentrations by direct and extraction-based assays were 68% and 14% higher than the results obtained with an MS/MS-based method. An advantage of high-performance liquid chromatography (HPLC)-MS/MS–based methods for steroid analysis is specific detection, but these methods often lack sufficient sensitivity for measurement of low endogenous concentrations in postmenopausal women, men, and prepubertal children. Recent approaches to high-sensitivity analysis of steroids in serum by liquid chromatography (LC)-MS/MS use derivatization to enhance detection.[22-26]

The need for a high-sensitivity, high-specificity mass spectrometry method for measuring estrogens was recently emphasized by Lee et al.[21] Two LC-MS/MS methods for measuring estrogens that use dansyl chloride derivatization and rapid chromatographic separation were published.[23,26] Dansyl chloride is highly reactive with compounds containing hydroxyl and amino groups. Because the derivatives have nonspecific fragmentation patterns, this causes poor analytic specificity. During the preliminary study of a method using this derivative, we detected numerous interferences coeluting with estrogens and internal standards (ISs) under fast LC separation conditions. This led us to evaluate and adopt a 2-dimensional (2D) separation, assessment of the specificity of analysis through monitoring multiple mass transitions,[27] and selection of isotopically labeled ISs for which there were no endogenous interferences.

Our intention was to develop a method suitable for measurement of endogenous concentrations of estrogens in postmenopausal women, men, and children, which, as shown later in this article, requires high analytic sensitivity and specificity that were lacking in earlier published methods.

Materials and Methods

Standards and Reagents

Standards of E_1 and E_2 (purity 98% or better) were purchased from Sigma, St Louis, MO. Deuterated analogs of the steroids d_4-E_1, d_3-E_2, and d_5-E_2 were purchased from CDN Isotopes, Pointe-Claire, Canada. Stock standards were prepared in methanol at concentrations of 1 g/L. Working combined calibration and ISs were prepared in a mix of water and methanol (1:1) at concentrations of 0.4 and 2.0 μg/L, respectively. Calibration standards of estrogens were prepared in 0.05% bovine serum albumin at concentrations of 5, 20, 50, 80, 120, and 200 pg/mL. HPLC-grade water, methanol, methyl t-butyl ether, and acetonitrile were obtained from VWR, West Chester, PA. All other reagents were purchased from Sigma and were of the highest purity commercially available. Certified reference materials (CRM) BCR-576, BCR-577, and BCR-578 were from the Institute for Reference Materials and Measurements, Geel, Belgium, and used for the evaluation of the accuracy of E_2 measurements.

Sample Preparation

Aliquots of 200 μL of standards, control samples, or patient serum were transferred into microcentrifuge tubes. To each tube, we added 20 μL of a working combined IS. The samples were extracted with 1.2 mL of methyl t-butyl ether; the organic was transferred into a 96-well plate, and evaporated under nitrogen at 50°C. The dried residues were redissolved in 50 μL of dansyl chloride (3.7 mmol/L) in a 1:1 mix of acetonitrile and aqueous sodium carbonate (10 mmol/L). The plate was vortex-mixed and incubated in a heating block at 60°C for 10 minutes. After the incubation, 50 μL of a mix of acetonitrile and water (1:1) was added to each sample, and the samples were analyzed.

Liquid Chromatography–MS/MS

The instrument consisted of an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Canada) with a TurboIonSpray ion source operated at 650°C. The system included a Shimadzu LC-10RD pump (Kyoto, Japan), a series 1200 Agilent HPLC pump and oven (Santa Clara, CA), and an HTC PAL autosampler (Carrboro, NC) equipped with a fast wash station and 10-port switching valve. The first dimension of separation was on a C_1 cartridge, and the analytic separation was performed on a 100 × 2.0 mm HPLC column Germini Phenyl with 3-μm particles (both from Phenomenex, Torrance, CA). The injection volume was 50 μL, and the oven temperature was 30°C.

For the first-dimension separation, the mobile phase consisted of water with 10 mmol/L of formic acid (bottle A1) and methanol with 10 mmol/L of formic acid (bottle B1). For the second-dimension separation, the mobile phase consisted of water with 10 mmol/L of formic acid (bottle A2), and in bottle B2 acetonitrile with 10 mmol/L of formic acid. The mobile phase for the first-dimension separation was delivered at a flow rate of 1 mL/min with a gradient: 10% methanol for 0.3 minute, linear gradient to 70% methanol between 0.3 and 1 minute, step gradient to 95% of methanol at 1.1 minutes, followed by reequilibration to initial conditions after 3 minutes; between 3 and 6 minutes, the flow rate was reduced to 0.2 mL/min. The mobile phase for the analytic separation was delivered at a flow rate of 0.6 mL/min with a gradient: 50% acetonitrile for 1.3 minutes, linear gradient to 85% acetonitrile between 1.3 and 6.8

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minutes, step gradient to 95% of acetonitrile at 6.9 minutes, followed by reequilibration to initial conditions between injections. A switching valve was installed between the columns; effluent from the C1 column was directed to the analytic column between 0.5 and 1.2 minutes.

The autosampler injection syringe was washed between injections 10 times with an acetonitrile-water mix (ratio, 4:1) containing 60 mmol/L of trifluoroacetic acid. The injection wash valve was washed after each injection with 3 mL of the same solvent mix. The quadrupoles Q1 and Q3 were tuned to unit resolution, and the mass spectrometer conditions were optimized for maximum signal intensity of each estrogen. The instrument was operated in positive-ion mode with ion spray voltage of 5,000 V and entrance potential of 10 V. Two mass transitions were monitored for each estrogen and its IS. The primary transitions were from parent ions to the product ion *m/z* 156 (collision energy, 80 V), and the secondary transitions were from the same parent ions to the product ion *m/z* 171 (collision energy, 55 V).

Nitrogen was used as the gas in the collision cell. All data were acquired and processed with Analyst 1.4.2 software (Applied Biosystems/MDS Sciex). A quantitative calibration was performed with every batch of samples.

Assay Performance Characteristics

Evaluation of method performance included imprecision, limit of detection, limit of quantitation, upper limit of linearity, method comparison, interference potential, recovery, carryover, and ion suppression. Imprecision was determined by analyzing 3 replicates per run of samples containing estrogens (human plasma supplemented with estrogens) at concentrations of 8, 30, 100, and 200 pg/mL in 1 run per day for a 5-day period. Linearity was evaluated by analyzing samples prepared at concentrations of estrogens between 10 and 10,000 pg/mL. Method sensitivity was determined by analyzing samples containing progressively lower concentrations of estrogens down to 0.5 pg/mL. A criterion of maintaining accuracy within ± 15%, imprecision (coefficient of variation) less than 20%, and a branching ratio of the mass transitions within ± 30% was used to determine the upper limit of linearity and limit of quantitation for the assay and for assessment of specificity in unknown samples.[27] The limit of detection was determined as the lowest concentration at which chromatographic peaks of the estrogens were present in both transitions at expected retention times and the signal/noise ratio was greater than 3. The recovery of the method was determined by analyzing patient samples containing 30 to 40 pg/mL of endogenous E_1 and of endogenous E_2 (E_1, 111-148 pmol/L; E_2, 110-147 pmol/L) spiked with 35 pg/mL of each E_1 and E_2 (n = 3). The observed difference was compared with the expected concentration.

To examine possible interfering substances, we analyzed more than 50 steroids and steroid metabolites **Table 1** and more than 3,000 random patient samples. A branching ratio of the transitions outside of ± 30% limits, broadening of the chromatographic peaks, split peaks, or an increase in the background were interpreted as potential interference.

Ion suppression was evaluated by analyzing extracted samples with concentrations of estrogens less than 20 pg/mL injected in flow of dansyl derivatives of E_1 and E_2 (each at 200 pg/mL) infused into the ion source of the mass spectrometer. A decrease in the intensity of the baseline in the mass transitions of the estrogens was considered as evidence of ion suppression.[28]

Table 1
Steroids and Steroid Metabolites Evaluated for Interference

1-(2-Chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane
17α-Methylandrostan-17β-ol-3-one
19-Nortestosterone 17-decanoate
3β-Hydroxy-5α-androstan-17-one
3β-Hydroxyetioallocholan-17-one
5α-Dihydrotestosterone
5-Androstenediol
5α-Dihydroprogesterone
5β-Dihydroprogesterone
11-Deoxycortisol
17-Hydroxyprogesterone
17-Hydroxypregnenolone
17α-Estradiol
Androstenediol
Androsterone
Cortisol
Cortisone
Cyproterone acetate
Danazole
Dihydroepiandrosterone
Epiandrosterone
Epitestosterone
Ethylestrenol
Ethynodiol diacetate
Etiocholan-17β-ol-3-one
Etiocholanolone
Fluoxymesterone
Flutamide
Hermaphrodiol
Isoandrosterone
Isotestosterone
Levonorgestrel
Medroxyprogesterone
Mesterolone
Methandrostenolone
Methenolone
Methylandrostanolone
Methyldihydrotestosterone
Methyltestosterone
Nandrolone
Norethandrolone
Norethindrone
Norprogesterone
Oxandrolone
Oxymetholone
Pregnenolone
Stanozolol
Testosterone
trans-Androsterone
trans-Dihydrotestosterone

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Reference Intervals, Specimen-Type Suitability, and Stability

We obtained serum samples for reference interval study from apparently healthy volunteers after obtaining informed consent. Serum was separated from RBCs within 1 hour after collection, and the samples were stored at –70°C before analysis. The participants were not taking prescription medications; women taking oral contraceptives or hormone replacement therapy were excluded from the study. More than 90% of participants were of Caucasian descent. The mean (median) body mass index (BMI) values were 21.3 (21.4), 25.3 (27.2), and 24.2 kg/m^2 (24.5 kg/m^2) for 81 premenopausal women, 40 postmenopausal women, and 113 men, respectively. The mean (median) ages of adult volunteers were 32.1 (30.0), 53.4 (53.9), and 31.8 years (35.3 years) for premenopausal women, postmenopausal women, and men, respectively. Samples from 7- to 17-year-old children (444 samples from girls and 325 samples from boys) were collected after obtaining parental permission. The Tanner stage (stage of sexual development) of each child was determined. Nonparametric reference intervals were determined as the central 95% range.

Stability of the steroids under different storage conditions was evaluated as follows. Aliquots of serum samples containing 130 to 250 pg/mL of E_1 and E_2 (E_1, 481-925 pmol/L; E_2, 477-918 pmol/L) were stored at room temperature in a refrigerator (4°C) and in a freezer (–20°C). The samples were transferred into a –70°C freezer after 1, 3, 14, 21, 30, and 60 days of storage and analyzed in a single batch. Blood from 12 volunteers was collected in serum gel separator and sodium EDTA tubes for assessment of specimen-type suitability. All studies with samples from human subjects were approved by the University of Utah Institutional Review Board.

Method Comparison

The LC-MS/MS method was compared with an in-house radioimmunoassay (RIA) for E_1 (n = 67; Diagnostic Systems Laboratories, Webster, TX), chemiluminescent immunoassay for E_2 (n = 41; Vitros ECi, Ortho-Clinical Diagnostics, Rochester, NY), extraction-based RIA (20 samples each for E_1 and E_2), and LC-MS/MS[29] methods (20 samples each for E_1 and E_2) of commercial laboratories. The results were evaluated by using Deming regression.[30]

Results

The chromatograms of 2 mass transitions of E_1 and E_2 extracted from serum of postmenopausal women are shown in **Figure 1**. Concentrations of E_1 and E_2 were determined using mass transitions to the product ion *m/z* 156 for each compound and its IS; these mass transitions proved to be more specific compared with *m/z* 171. Ratios of concentrations determined from corresponding primary and secondary mass transitions for each estrogen and its IS were calculated to evaluate the specificity of the analysis.[27,31] The acceptance limit for the ratio was set at ± 30%.

The limits of quantitation and detection for E_1 and E_2 were 1 pg/mL and 0.5 pg/mL, respectively. The assays were linear up to 5,000 and 10,000 pg/mL for E_1 and E_2, respectively. Signal/noise ratios at a concentration of 6 pg/mL (0.3 pg

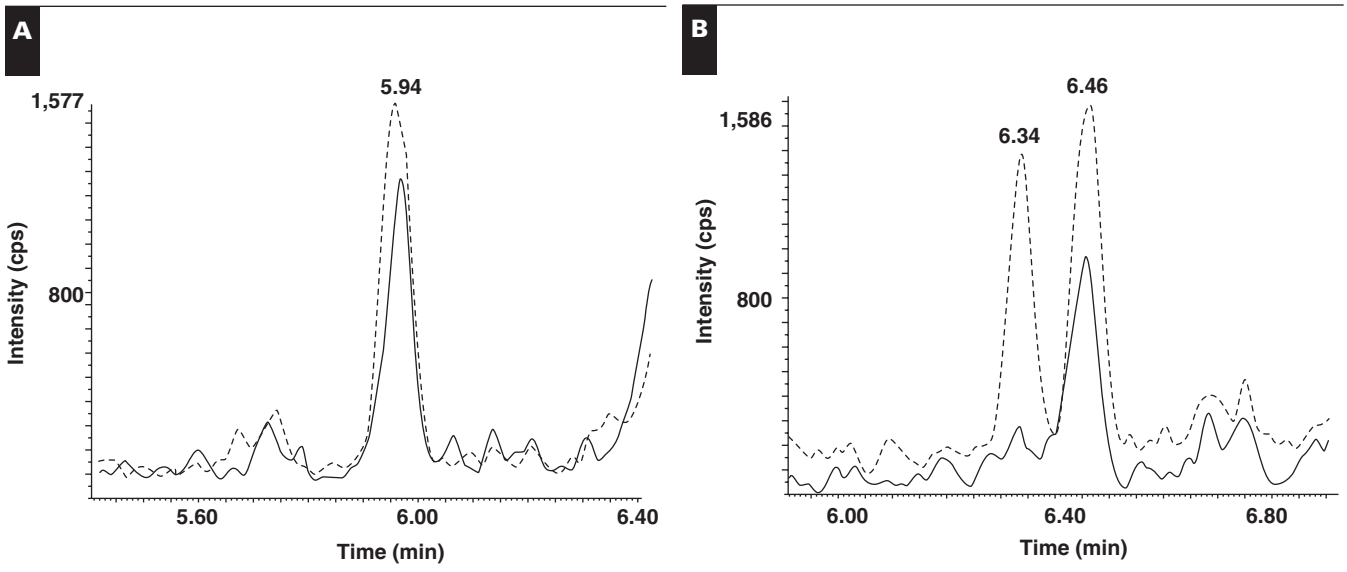

Figure 1 Multiple reaction monitoring chromatograms (overlay of the product ions *m/z* 156, solid line; *m/z* 171, dashed line) of extracted patient serum sample containing 14 pg/mL (52 pmol/L) of estrone (**A**) and 19 pg/mL (70 pmol/L) of estradiol (**B**).

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injected onto the HPLC column; n = 5) for E_1 and E_2 were 18 and 15, respectively; the recoveries for E_1 and E_2 were 95%. Within-run, between-run, and total imprecision data obtained are shown in **Table 2**. The results of the method comparison with commercial immunoassays and methods of commercial laboratories are shown in **Figure 2**. The measured concentrations of E_2 in 3 CRM samples (31, 187, and 364 pg/mL) were within 90.0%, 97.9%, and 99.2%, respectively, of the target values.

More than 50 steroids and steroid metabolites (Table 1) were evaluated for potential interference with the method. The steroids were analyzed at concentrations of 100 µg/L, and the chromatograms were evaluated for the presence of peaks at the mass transitions of estrogens. None of the steroids evaluated except 17αE_2 interfered with the method. 17αE_2 was not resolved from 17βE_2 by the chromatographic conditions used in the assay and produced identical mass transitions. We were able to resolve the 17αE_2 and 17βE_2 by using a biphenyl column (Restek, Bellefonte, PA), but the analysis time was more than double that of the current method.

The estrogens showed no degradation under the storage conditions evaluated; no changes in concentrations of the estrogens were observed after 3 freeze-thaw cycles. No significant difference in the recovery was observed between serum and EDTA plasma samples. Evaluation of the suppression showed a drop in the baseline at retention times of 1.3, 1.8, and 4.3 minutes; no ion suppression was observed at the retention times of the estrogens.

Results of the analysis of the samples from volunteers grouped by Tanner stage and age are summarized in **Table 3** and **Table 4**. In girls, estrogen concentrations started rising at age 9 years and reached a maximum by age 14 years (median of total $E_1 + E_2$, 80.5 pg/mL [298 pmol/L]); in boys the concentrations started rising after age 12 years and reached a maximum by age 15 years (median of total $E_1 + E_2$, 39.3 pg/mL [146 pmol/L]). Plots of median concentrations of E_1 and E_2 and the median E_2/E_1 ratios by age in females and males are shown in **Figure 3**. The median ratio in girls was 1.78 (age 9-17 years) and in boys was 1.08 (age 12-17 years). Concentrations of estrogens in both sexes reached adult levels by Tanner stage 3. Peak values for the ratio were observed at ages 13 and 15 years in girls and boys, respectively. In postmenopausal women, the median concentration of total $E_1 + E_2$ was 22 pg/mL (81 pmol/L), and the median E_2/E_1 ratio was 0.55; in men, the median concentration was 39 pg/mL (144 pmol/L), and the median ratio was 0.98. The median E_2/E_1 ratio in females of reproductive age (18 years or older) was 1.49 (1.30 [n = 11], 1.56 [n = 23], and 1.48 [n = 37] during early follicular, late follicular, and luteal stages of the menstrual cycle, respectively).

In women of reproductive age, concentrations of estrogens and the E_2/E_1 ratio through the menstrual cycle were not age-dependent. No statistically significant changes in concentrations of estrogens and E_2/E_1 ratios were observed with age (48-63 years) in 40 postmenopausal women. In 113 men between the ages of 18 and 60 years, the E_1 concentration was age-dependent with the linear regression fit line $E_1 = 13.7 + 0.18 * \text{Age}$ ($r = 0.269$; $P = .0044$), whereas the concentration of E_2 and the E_2/E_1 ratio were not age-dependent; no statistically significant correlations between BMI and concentrations of E_2, E_1, and the E_2/E_1 ratio were found.

Table 2
Within-Run, Between-Run, and Total Imprecision of the Method

Sample	Mean, pg/mL (pmol/L)	Coefficient of Variation (%)		
		Within-Run	Between-Run/d	Total
Estrone				
Level 1	7.82 (29)	9.08	4.47	10.12
Level 2	16.71 (62)	4.89	8.45	9.76
Level 3	77.17 (285)	5.80	7.67	9.62
Level 4	208.93 (773)	4.44	6.85	8.17
Estradiol				
Level 1	8.37 (31)	9.31	6.94	11.62
Level 2	17.92 (66)	8.18	5.33	9.76
Level 3	77.28 (284)	4.25	7.03	8.22
Level 4	205.80 (756)	5.17	4.81	7.06

Discussion

The 3 approaches for analyzing estrogens using MS detection evaluated during method development included the following: (1) use of electrospray ionization with detection of deprotonated molecular ions in the negative ion mode with postcolumn addition of proton acceptors, (2) atmospheric pressure chemical ionization in the positive ion mode using protonated dehydrated estrogens as molecular ions, and (3) derivatization of estrogens with amine-containing sulfonyl halides and detection with electrospray ionization in positive ion mode.[23,26] The first 2 approaches did not provide sufficient sensitivity for measurement of estrogens at concentrations less than 10 pg/mL.

The yield of the derivatization reaction was highly dependent on the conditions under which it was performed. Optimization of the reaction using experimental design methods[32] showed that the concentration of dansyl chloride, the pH of solution, the incubation temperature, and the incubation time had a strong impact on the reaction recovery. The recovery improved with increased concentrations of dansyl chloride and in basic solution (pH 10-11). The recovery was adversely affected by extended exposure to elevated temperature and extended incubation time.

The advantages of dansyl chloride as the derivatizing reagent for estrogens include significant gains in sensitivity

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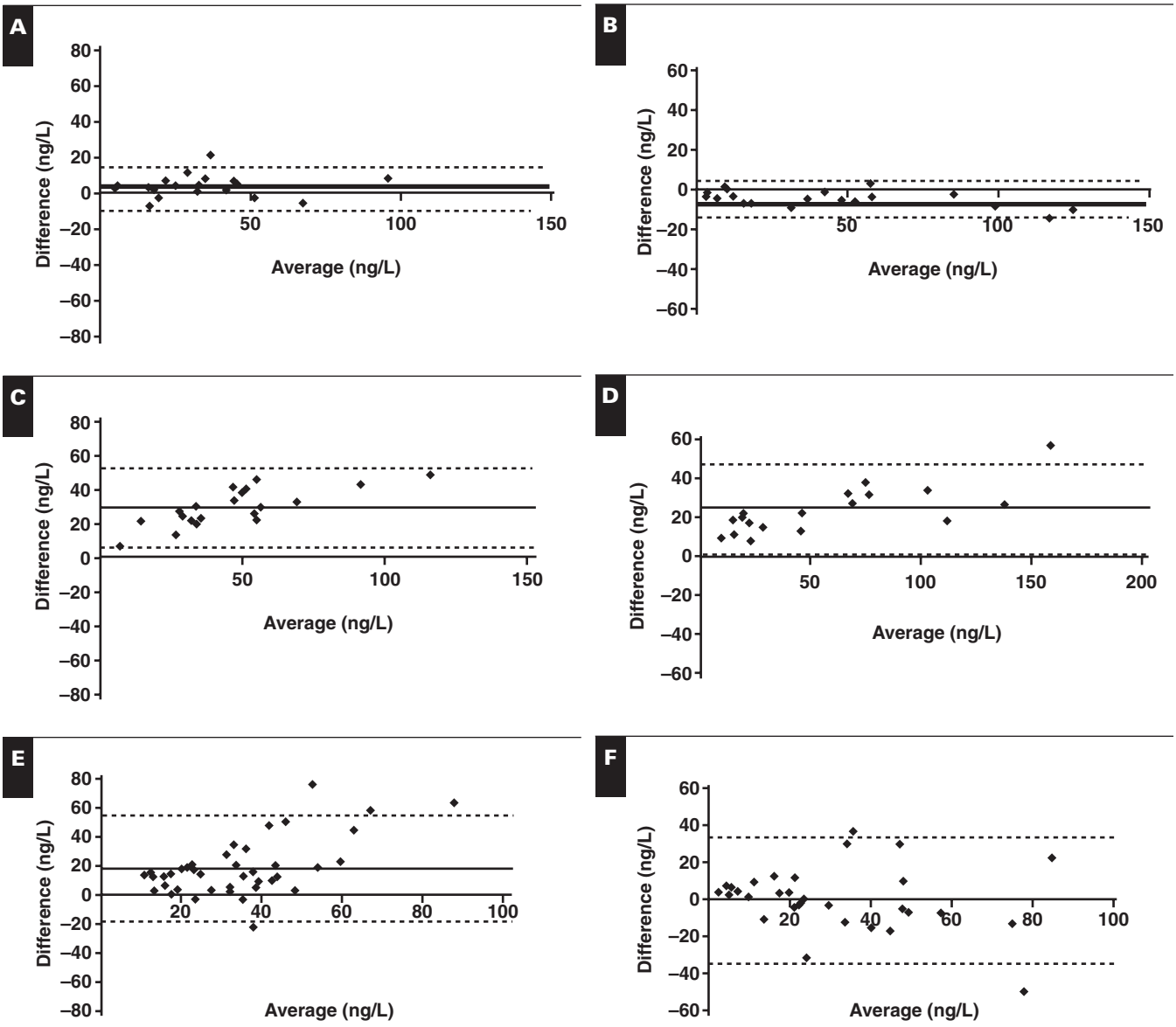

Figure 2 Results of the method comparison for estrone (**A**, **C**, and **E**) and estradiol (**B**, **D**, and **F**) with liquid chromatography–tandem mass spectrometry methods of commercial laboratory (**A** and **B**), extraction radioimmunoassay methods of commercial laboratory (**C** and **D**), and commercial direct immunoassays (**E** and **F**). Solid lines, means of 2 methods; dotted lines, ± 2 SD. **A**, y = 0.98 * x + 4.56; r = 0.959; $S_{y/x}$ = 6.1. **B**, y = 0.94 * x + 2.0; r = 0.995; $S_{y/x}$ = 3.7. **C**, y = 1.34 * x + 18.7; r = 0.961; $S_{y/x}$ = 6.0. **D**, y = 0.94 * x + 2.0; r = 0.995; $S_{y/x}$ = 3.7. **E**, y = 1.03 * x + 18.0; r = 0.590; $S_{y/x}$ = 11.1. **F**, y = 0.65 * x + 11.3; r = 0.743; $S_{y/x}$ = 17.5. Conversion of conventional units to Système International units (pmol/L) are as follows: estrone, multiply by 3.698; estradiol, multiply by 3.671.

and mild reaction conditions. The main disadvantage of the reagent is nonspecific fragmentation of the derivatives: all dansyl derivatives produce the same major product ions at *m/z* 156 and *m/z* 171. The ion *m/z* 171 originates from a cleavage of a C-S bond in the dansyl portion of the molecule, and the ion *m/z* 156 is produced by loss of the methyl group from the *m/z* 171. Another complication in the analysis is that the second isotopic ion (A + 2) of the molecular ion of dansyl E_1 (DE_1) is an isobar to the molecular ion of dansyl E_2 (DE_2). The presence of a sulfur atom within this structure causes the second isotope (A + 2) to become more intense and results in interference with DE_2, if peaks of DE_1 and DE_2 are not chromatographically resolved. In this method, DE_1 and DE_2 peaks were chromatographically resolved to avoid these problems. Dansyl chloride is highly reactive with hydroxyl- and amine-group–containing compounds,

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Table 3
Reference Intervals for Estrogens in Serum of Males by Tanner Stage and Age

	No. of Samples	Estrone (E_1), pg/mL (pmol/L)	Estradiol (E_2), pg/mL (pmol/L)	Total Estrogens ($E_1 + E_2$), pg/mL
Tanner stage				
1	134	<7 (26)	<8 (29)	<11
2	60	<10 (37)	<9 (33)	<19
3	53	1-31 (4-115)	1-35 (4-129)	3-48
4 and 5	74	2-30 (7-111)	3-35 (11-129)	4-64
Age group (y)				
7-9	94	<6 (22)	<6 (22)	<9
10-12	95	<10 (37)	<10 (37)	1-19
13-15	81	1-30 (4-111)	1-36 (4-132)	3-62
16-17	49	1-32 (4-118)	3-34 (11-125)	4-64
18-61	113	9-36 (933-133)	8-42 (29-154)	19-69

Table 4
Reference Intervals for Estrogens in Serum of Females by Tanner Stage and Age

	No. of Samples	Estrone (E_1), pg/mL (pmol/L)	Estradiol (E_2), pg/mL (pmol/L)	Total Estrogens ($E_1 + E_2$), pg/mL
Tanner stage				
1	158	<26 (96)	<55 (202)	1-86
2	75	1-39 (4-144)	2-133 (7-488)	3-169
3	100	8-117 (30-433)	12-277 (44-1,017)	23-361
4 and 5	108	4-109 (15-403)	2-259 (7-951)	8-341
Menarche				
Before	236	<41 (152)	1-84 (4-308)	2-119
After	205	4-113 (15-418)	3-264 (11-969)	10-337
Age group (y)				
7-9	65	<25 (93)	<35 (129)	<49
10-12	120	<42 (155)	<87 (319)	2-118
13-15	127	8-105 (30-388)	9-248 (33-910)	15-332
16-17	129	4-133 (15-492)	2-266 (7-977)	6-355
Postmenopausal women				
41-63 y	40	3-32 (11-118)	2-21 (7-77)	5-52

and this makes the method very sensitive to endogenous and exogenous impurities present in samples.

Initially, the deuterium-labeled analog d_5-E_2 was used as an IS for E_2. An interfering peak partially coeluting with d_5-E_2 at the mass transition *m/z* 511 to 171 was observed in approximately 30% of the samples; when the IS was changed to d_3-E_2, no interference was observed in the mass transition *m/z* 509 to 171. Such interference with the IS would cause underestimation of the E_2 concentration. This is particularly a concern in methods using fast chromatographic separation.[23,26] Because of nonspecific fragmentation of the dansyl estrogens, chromatographic separation has an important role in this method. The best selectivity for chromatographic separation was observed using a phenyl modified-stationary phase with retention of dansyl derivatives based on π-π interactions.

The nonspecific fragmentation of the dansyl derivatives of estrogens and low endogenous concentrations require highly selective sample preparation. Approaches that were evaluated for sample preparation included the use of various organic solvents, solid phase extraction (SPE), and liquid/liquid extraction followed by 2D chromatographic separation. Comparison of sample preparation using SPE on adsorbent Strata X (Phenomenex) and solvent extraction showed good absolute recovery with SPE but better signal/noise ratios for liquid/liquid extraction. The choice of extraction solvent allowed some reduction in the background noise, but the most improvement in the signal/noise ratio and elimination of the interfering peaks was achieved with 2D separation using C_1 phase as the first-dimension separation. Use of a 2D separation resulted in approximately 70% reduction of the background noise and improvement in sensitivity and specificity **Figure 4**. The first-dimension separation retained estrogens and passed through the majority of the impurities. After valve switching, the estrogens were back-flushed from the precolumn onto the analytic column and separated using gradient elution.

Method comparisons for E_1 and E_2 showed good agreement with LC-MS/MS assays performed by a commercial

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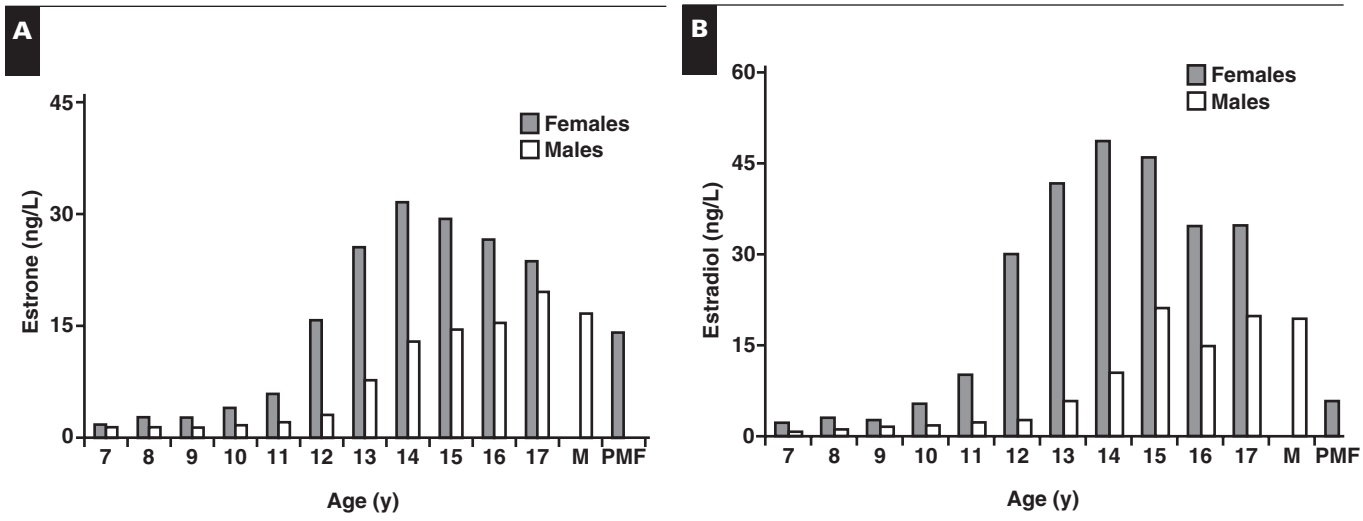

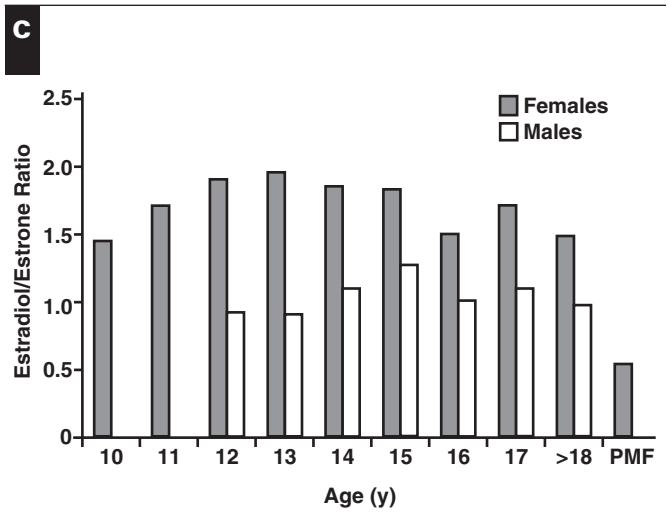

Figure 3 Median concentrations of estrone (**A**) and estradiol (**B**) and the estradiol/estrone ratio (**C**). The ratio was not calculated for 10- and 11-year-old boys. M, men (18 years and older), PMF, postmenopausal women.

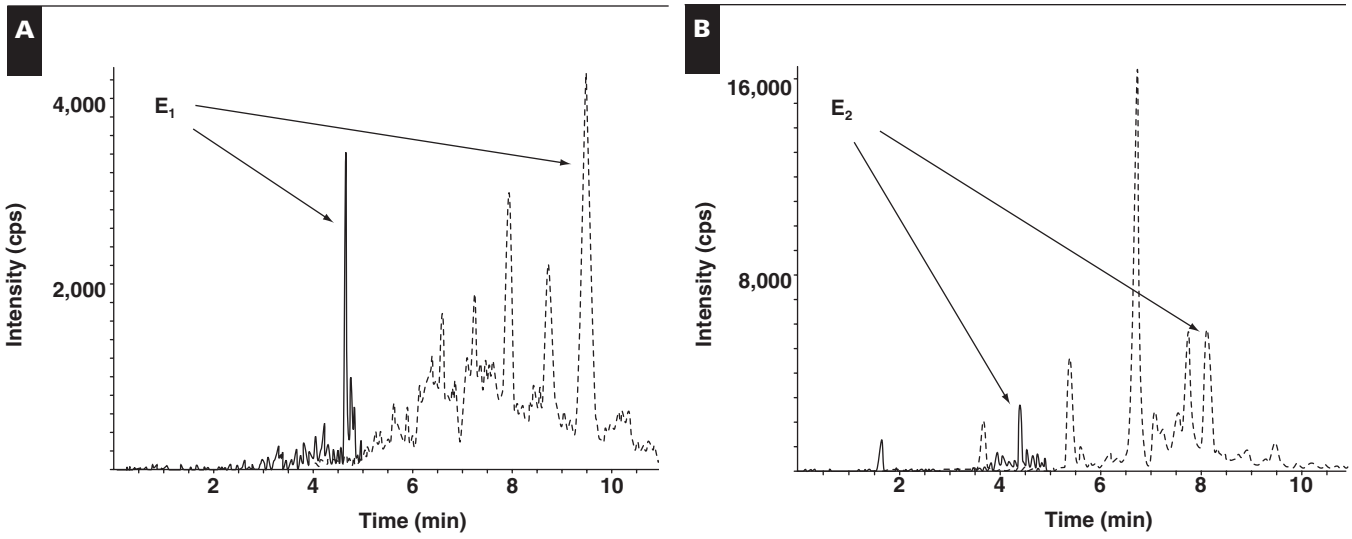

Figure 4 Patient serum sample analyzed for estrone (**A**, E_1) and estradiol (**B**, E_2) with chromatographic separation using a single analytical column (1D; dashed line) and 2-dimensional (solid line) separation. The 1D separation was performed using a shallow gradient with a 10-minute analysis time.

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laboratory[29] (Figure 2). The comparison LC-MS/MS methods used larger sample volumes, and the E_1 comparison method was less sensitive than our method. Other published LC-MS/MS methods[23,26] using dansyl chloride derivatization and fast chromatographic separation were also less sensitive and were affected by interference. Figure 4 shows an overlay of chromatograms of a patient serum sample analyzed by a single analytic column (1D) and a 2D separation. The chromatogram corresponding to the 1D separation was obtained using a shallower gradient than the gradients used in published methods.[23,26] Multiple peaks were resolved from E_1 and E_2 using this shallower gradient. Some of these resolved peaks would coelute with E_1 and E_2 when using a steeper gradient in a fast separation. If an interfering peak coeluted with E_1 and E_2, a false elevation in concentration would occur. If the interfering peak coeluted with one of the internal standards, a false decrease in concentration would occur. Because the frequency of such interferences in clinical samples is high, a rapid 1D method is not suitable for testing samples with low concentrations of estrogens. In our method, the 2D chromatographic separation eliminates the interfering peaks and significantly reduces the background noise, leading to an increase in sensitivity and elimination of potential interferences (Figure 4). In addition, the 2D separation takes less time than would be required for a good quality 1D separation.

The E_2 concentration observed in 3 CRM samples analyzed during the method validation agreed with expected values. Comparison of our method with direct immunoassay showed large discrepancies between the methods for both E_1 and E_2 (Figure 2). The scatter was lower for the extraction-based RIA methods, but direct immunoassay and the extraction-based RIA had constant and proportional biases and were less sensitive compared with our LC-MS/MS method. The high sensitivity of our method and its good agreement with other LC-MS/MS methods from a commercial laboratory[29] suggest that this method is suitable for measurement of estrogens in populations with low endogenous concentrations of estrogens. The improved sensitivity and specificity of our method are likely partial explanations for differences in the reference intervals determined in an earlier LC-MS/MS method.[26] Differences in the reference intervals could also be attributed to different reference populations. Because of a lack of sensitive and specific methods for measurement of estrogens, limited information on pediatric reference intervals was available in the past.[6,7] Reference intervals that we determined for postmenopausal women and children (Tables 3 and 4) demonstrate the need for measurements of E_1 and E_2 concentrations as low as 1 pg/mL (4 pmol/L for E_1 and E_2).

We developed a highly sensitive method for analysis of estrogens in serum. In our experience, the best sensitivity and selectivity for estrogens was achieved using dansyl chloride derivatization in conjunction with a 2D chromatographic separation. Based on the high sensitivity and specificity, the method is suitable for measurement of estrogens in samples from postmenopausal women, men, and children. We established reference intervals for E_1 and E_2 for males and females of different Tanner stages and age groups using an isotope dilution LC-MS/MS method. The small sample volume required for this test helps reduce the volume of blood obtained from patients, making it especially useful for pediatric testing.

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