


Ultrasensitive Semiautomated Chemiluminescent Immunoassay for Estradiol, Barry G. England,1 George H. Parsons,2† Russell M. Posley,3 Daniel S. McConnell,1 and A. Rees Midgley1 (The University of Michigan, Clinical Ligand Assay Satellite Services [CLASS Laboratory and Study of Women’s Health Across the Nation (SWAN)], 1301 Catherine St., Medical Science I, M3232A, The University of Michigan, Ann Arbor, MI 48109;2 Bayer Diagnostics, 333 Coney St., East Walpole, MA 02082;3 current address: Future Diagnos

The availability of rapid, non-extraction-based estradiol-17β immunoassays has been important in clinical settings where prompt turnaround time is required, including in vitro fertilization programs, and in large-scale research programs where large numbers of samples are processed. However, the inaccuracy of estradiol-17β assay results has been recognized for some time, as indicated in the Textbook of Reproductive Medicine by L.R. Boots (1), in which he states: “Everyone measures estradiol levels and it is probably assumed that few if any problems exist with this assay. . . .” The most common use of estradiol levels is in relation to levels of estradiol during ovulation stimulation and every assay provides clinically relevant results. These results are clearly inaccurate but usually precise (1). In the First and Second E2 International Workshops (2), problems associated with the analysis of estradiol-17β were fully discussed. The First E2 International Workshop focused on the need for more specific, precise, and sensitive estradiol-17β assays, and the Second E2 International Workshop explored various means of increasing accuracy in the measurement of estradiol-17β. By consensus, the use of a panel of 22 samples confirmed by isotope dilution–gas chromatography–mass spectrometry (ID-GC-MS) (2) was made available for use in assay development. Indications of a lack of accuracy are also provided by examining survey programs, such as those offered by the College of American Pathologists, that reveal large differences in estradiol-17β values obtained on the same sample.

Modern serum assays for estradiol-17β claim detection limits as low as 40 pmol/L or lower, but they lack sufficient sensitivity for pediatric or postmenopausal specimens and are frequently unable to provide adequate analytical precision and accuracy in samples from human males. In postmenopausal women, circulating estradiol-17β is usually <73 pmol/L, and concentrations ≤18 pmol/L are common. Circulating estradiol-17β in men is somewhat higher, but concentrations >147 pmol/L are rare. In prepubertal children, estradiol-17β is even lower than in postmenopausal women (3). We describe a new method that uses ID-GC-MS-confirmed calibrators with a sensitivity requisite to measure estradiol-17β in samples with low concentrations of this analyte.

The assay was developed on an ACS-180 system equipped with the manufacturer’s software and using E2-6 reagents supplied by Bayer Diagnostics. Calibrators and antibody were diluted in Bayer Multidiluent Buffer (MD-9) before the incubation scheme described below. Briefly, assay sensitivity is increased by allowing the estradiol-17β in 400 μL of a sample (calibrator):MD-9 (1:1) mixture to bind to antibody in 400 μL of an anti-E2-6:MD-9 (1:3) mixture during a 30-min (37°C) offline incubation. After the incubation, 200 μL of the calibrator or sample-antibody mixture is aspirated by the sample probe and added to the onboard incubation cuvette. Estradiol-17β, labeled with dimethyl-acridinium ester (E2-DMAE), and mouse anti-rabbit immunoglobulin G conjugated to superparamagnetic particles (PMPs) are added to the incubation cuvette at probe 2. The E2-DMAE occupies free binding sites on the anti-E2-6, and the PMP-bound mouse anti-rabbit immunoglobulin G immobilizes the antibody. The immobilized anti-E2-6-labeled estradiol complex is pulled to the sides of the incubation cuvette by a set of rare earth magnets, and unbound E2-DMAE is washed out of the cuvette. Emission of light by the PMP-antibody-bound acridinium ester trace is then induced by the addition of hydrogen peroxide at a low pH to the cuvette, followed by the addition of sodium hydroxide, which releases the peroxide anion and initiates the light emission reaction. An inverse relationship exists between estradiol-17β concentrations in the sample and relative light units detected by the ACS-180. Calibrators containing estradiol-17β at 1.8, 3.7, 7.3, 14.7, 29.4, 45.9, 91.8, 183.5, 367.1, and 917.8 pmol/L were prepared from the Bayer estradiol-17β Master Curve Material. Data analysis was performed using the StatLIA® statistical ligand immunoassay analysis software (Brendan Scientific Corp.) with the StatLIA four-parameter symmetrical logistic function.

The accuracy of the assay was evaluated by comparing two ID-GC-MS-confirmed samples, samples 19 and 20 (2), with Bayer-supplied estradiol-17β calibration material. Within-run imprecision was evaluated by comparing results for samples obtained from premenopausal, perimenopausal, and postmenopausal women, assayed in duplicate in one of four independent assays. The reproducibility of the method was assessed by repeated measurements of low concentrations of estradiol-17β in male serum. Five replicates of diluted (1:2) and undiluted samples were analyzed in duplicate in each of four
different assays (20 duplicate results for each individual). The Fisher LSD (Protected t-test) statistical procedure revealed significant between-subject differences ($P < 0.001$) but no significant within-subject differences. Aldosterone, cholesterol, cortisone, cortisol, danazol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, estradiol, estradiol glucuronate, estril, estrone, progesterone, corticosterone, testosterone, norethindrone, pregnenolone sulfate, 11-deoxycortisol, 17a-hydroxyprogrenenolone, and 17a-hydroxyprogesterone at concentrations of $10^{-5}$–$10^{-4}$ pmol/L demonstrated no significant cross-reactivity under assay conditions ($<0.001$% cross-reactivity). Recovery studies performed by adding 18, 92, and 184 pmol/L estradiol-17β to MD-9 yielded a mean recovery of 104% (97%, 97%, and 117%, respectively), with the greatest variability observed on the addition of 18 pmol/L.

The clinical utility of this assay was confirmed by comparing daily serum estradiol-17β concentrations in 11 cycling women (Fig. 1) with daily urinary estrone conjugate concentrations measured by the method of Munro et al. (4). Daily results were indexed by the day of peak serum luteinizing hormone to permit comparison of estrone and estradiol-17β results during luteal and follicular phases of the menstrual cycle.

The estradiol calibration curve (1.8–918 pmol/L) was linear on a logit-log plot, and the 50% point of 102 pmol/L (75 assays) had a CV of 6.1%. All points were significantly different from each other (ANOVA, $P < 0.001$), and the lowest calibrator (1.8 pmol/L) was significantly different ($P < 0.01$) from the 0.0 pmol/L calibrator.

Results from the two ID-GC-MS-confirmed samples (samples 19 and 20) compared favorably with calibrators from Bayer Diagnostics. Measured results for sample 19 (367 pmol/L) were 93%, 99%, and 91% of expected at dilutions of 1:1, 1:2, and 1:4, respectively. For sample 20 (367 pmol/L), they were 96%, 99%, and 94%, respectively, at the same dilutions.

Within-run CVs were $<10\%$ except at extremely low concentrations. Grouping of samples in 36.7 pmol/L bins revealed that all bins had CVs $<10\%$, except in the 0.0–36.7 pmol/L bin, where the CV was 12.5%. ANOVA confirmed the uniformity of variance in all other bins. Cross-reactivity was $<1\%$ for estril (0.28%), estrone (0.75%), 17α-estradiol (0.04%), estradiol glucuronide (0.09%), and aldosterone (0.05%). All others showed $<0.009\%$ cross-reactivity in the assay. The low concentrations of estril and estrone found in the serum of non-pregnant women render the cross-reactivity of these steroids inconsequential in the current assay. Recovery of estradiol-17β added to serum pools with measured concentrations of 0, 20.93, and 102.42 pmol/L averaged 104%. Inter- and intraassay imprecision, evaluated with quality-control materials, showed similar CVs across the range of the assay with higher CVs at low concentrations of estradiol-17β. In most cases the CVs were $<10\%$, even in postmenopausal serum and serum from men, in which measured concentrations were $<33$ and $<76$ pmol/L, respectively, the CVs were $<12.5\%$.

Serum estradiol-17β and urinary estrone conjugates showed the cyclical changes expected in women with normal menstrual cycles: a dramatic increase in circulating concentrations during the midcycle preovulatory peak and secondary increases during the luteal and follicular phases. Creatinine-adjusted urinary estrone conjugates lagged behind the circulating concentration of the parent steroid by $\sim 1$ day throughout the cycle and appeared to lag by $\geq 2$ days during the midcycle increase in estradiol-17β. The cause of the additional lag time noted during the preovulatory surge of luteinizing hormone and estradiol-17β is not known.

Variability in estradiol-17β measurement, as cited in the literature by Lee et al. (5) and revealed by a careful examination of the College of American Pathologists survey results, confirms that the performance characteristics of direct immunoassays for estradiol-17β need to be improved, particularly with respect to analytical accuracy and detection limit. The use of weighed calibrators or the availability of a panel of samples with known concentrations of estradiol-17β (2) could enhance accuracy of the various estradiol-17β immunoassay methods. Assay sensitivity is particularly important in the measurement of estradiol-17β in normal serum samples in which physiologic concentrations of total estradiol-17β can be expected to range between 4 and 367 pmol/L.

Most commercially available estradiol-17β immunoassays today have detection limits between 73 and 183 pmol/L (5). This makes it difficult or impossible to measure the circulating concentrations (6–8) of this analyte in postmenopausal women, men, and children. The sensitive estradiol-17β immunoassay method described here fully meets our need for rapid, accurate, and precise analysis of serum estradiol-17β. The assay procedure uses an antibody with high-specificity binding characteristics, confirmed the uniformity of variance in all other bins. Cross-reactivity was $<1\%$ for estril (0.28%), estrone (0.75%), 17α-estradiol (0.04%), estradiol glucuronide (0.09%), and aldosterone (0.05%). All others showed $<0.009\%$ cross-reactivity in the assay. The low concentrations of estril and estrone found in the serum of non-pregnant women render the cross-reactivity of these steroids inconsequential in the current assay. Recovery of estradiol-17β added to serum pools with measured concentrations of 0, 20.93, and 102.42 pmol/L averaged 104%. Inter- and intraassay imprecision, evaluated with quality-control materials, showed similar CVs across the range of the assay with higher CVs at low concentrations of estradiol-17β. In most cases the CVs were $<10\%$, even in postmenopausal serum and serum from men, in which measured concentrations were $<33$ and $<76$ pmol/L, respectively, the CVs were $<12.5\%$.

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has no extraction requirements, and preserves most of the
advantages of automation.

In summary, we report the development of a highly sensitive and precise semiautomated immunoassay that is capable of measuring estradiol-17β concentrations in women during the perimenopausal and postmenopausal intervals and in healthy men and children.

References

Iron-Replete Reference Intervals to Increase Sensitivity of Hematologic and Iron Status Laboratory Tests in the Elderly, Timo I. Takala,1* Pauli Suominen,1 Raimo Isoaho,3,4 Sirkka-Liisa Kivelä,5,6 Minna Loppönen,4 Olli Peltola,1 Allan Rajamäki,2 and Kerttu Irjala1 (Departments of 1Clinical Chemistry and 2Hematology, TUCH—Laboratories, Turku University Central Hospital, PO Box 52, FIN-20520 Turku, Finland; 3Institute of Clinical Medicine, General Practice, University of Turku, FIN-20014 Turku, Finland; 4Härmätie Health Center, PO Box 51, FIN-21421 Lieto, Finland; 5Satakunta Central Hospital, Sairaalantie 3, FIN-28500 Pori, Finland; * author for correspondence: fax 358-2-3133920, e-mail Timo.Takala@tyks.fi)

In recent years both the concept and diagnosis of iron deficiency (ID) have evolved considerably. The introduction of new laboratory tests, especially soluble transferrin receptor (sTfR), has enabled the identification of storage iron depletion, iron-deficient erythropoiesis, iron deficiency anemia (IDA), and functional ID as readily distinguishable clinical conditions (1–5). Efficient use of the new tests, however, requires that the respective reference populations be appropriately characterized. A problem of conventionally derived reference intervals (RIs) is that the reference groups may contain individuals with subclinical ID (6). Especially in populations with an increased risk of ID, this may widen RIs and impair the sensitivity of these laboratory tests (7).

Efforts have been aimed at modeling the development of subclinical ID and, subsequently, toward establishing truly health-related RIs for adults, children, and adolescents (2, 7–11). Studies performed in the elderly have used highly variable criteria for the selection of reference populations (12–23). Therefore, despite the particular clinical interests regarding iron status in the elderly, no comprehensive studies covering the issue of subclinical ID are available.

This study was undertaken to investigate how subclinical ID influences the RIs of hematologic indices, sTfR, and other laboratory tests defining iron status in the elderly. To achieve this, we selected a general reference group of individuals ≥65 years of age and produced RIs for hematologic indices, sTfR, and other laboratory tests. We also formed an iron-replete subgroup by excluding individuals with storage iron depletion. The differences between values from the general reference group and the iron-replete subgroup were used to investigate the extent of storage iron depletion and its impact on the RIs of the studied tests.

This study was a part of a larger Lieto study and was carried out in Lieto, a semi-industrialized rural community in southwestern Finland. A total of 1260 (82%) elderly Lieto residents born during or before 1933 were enrolled (533 men and 727 women). The participants were interviewed and clinically examined. Their diseases were recorded with the diagnosis codes listed in the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10), and the medications were recorded. Venous blood samples were taken after an overnight fast from all participants. Written informed consent was obtained from all participants, and the Joint Commission on Ethics of the Turku University and Turku University Central Hospital approved the study protocol.

We selected retrospectively a general reference group according to the recommendations of the IFCC (24). In this group, we included those individuals who did not have diseases considered to interfere with hematologic indices, sTfR, and other laboratory tests (detailed exclusion criteria can be found in Table 1A, which is available as a data supplement with the online version of this Technical Brief at http://www.clinchem.org/content/vol48/issue9/). In addition to several diagnoses, we used increased C-reactive protein (≥10 mg/L) and creatinine (≥125 μmol/L in women and ≥135 μmol/L in men) concentrations as separate exclusion criteria for the reference group. The only medication we used to exclude individuals from the general reference group was iron supplementation. The iron-replete subgroup was defined from the general reference group by including only individuals with serum ferritin values ≥22 μg/L (8). Separate RIs were calculated for the general reference group and the iron-replete subgroup.

Hemoglobin (Hb), hematocrit, erythrocyte count, mean corpuscular Hb, and mean corpuscular volume were measured on an automated analyzer (Advia 120; Bayer Diagnostics). Plasma sTfR assays were performed by use