

## Validity of Free Testosterone and Free Estradiol Determinations in Serum Samples from Postmenopausal Women by Theoretical Calculations

Sabina Rinaldi, Annabelle Geay, Henri Déchaud, Carine Biessy, Anne Zeleniuch-Jacquotte, Arslan Akhmedkhanov, Roy E. Shore, Elio Riboli, Paolo Toniolo, and Rudolf Kaaks<sup>1</sup>

IARC, Unit of Nutrition and Cancer, 69500 Lyon, France [S. R., C. B., E. R., R. K.]; Service de Radioanalyse et Radiopharmacie, Hôpital Neurocardiologique, Hospices Civils de Lyon, Lyon, France [A. G., H. D.]; and Departments of Environmental Medicine [A. Z.-J., A. A., R. E. S., P. T.] and Obstetrics and Gynecology [A. A., P. T.], New York University School of Medicine, New York, New York 10010

### Abstract

**In this study, we validated measurements of free testosterone (fT) and free estradiol (fE<sub>2</sub>) concentrations calculated from total serum concentrations of testosterone (T), estradiol (E<sub>2</sub>), and sex hormone-binding globulin (SHBG), measured by direct, commercial radioimmunoassays, by comparison with reference measurements obtained by dialysis plus an in-house radioimmunoassay after extraction and chromatographic purification. The study was conducted in serum samples from 19 postmenopausal women who were part of an ongoing prospective cohort study. We also performed sensitivity analyses to examine the robustness of the theoretical calculations. Sensitivity analyses showed that in this population, competitive binding of dihydrotestosterone and total T could be ignored in the calculation of fE<sub>2</sub>, and competitive binding by dihydrotestosterone does not need to be taken into account for calculation of fT. Furthermore, variations in albumin and SHBG concentrations had negligible effects on fT and fE<sub>2</sub> calculations. Values of fT and fE<sub>2</sub>, calculated from total T and E<sub>2</sub> concentrations obtained by the same in-house radioimmunoassay used for the dialysis method, correlated highly with the measurements by dialysis (Pearson's coefficients of correlation above 0.97). When calculating fT and fE<sub>2</sub> using total T and total E<sub>2</sub> concentrations obtained by different direct radioimmunoassays, almost all kits gave good correlations with the reference method for fT (Pearson's  $r > 0.83$ ), but only a few gave good correlations for fE<sub>2</sub> (Diagnostic System Laboratories and DiaSorin;  $r > 0.80$ ). The direct radioimmunoassays giving the best correlation for fT and**

**fE<sub>2</sub> with the dialysis method were those that best measured total concentrations of T and E<sub>2</sub>. Furthermore, mean values of fT and fE<sub>2</sub> corresponded well to mean values by the reference method if SHBG measurements were also well calibrated. We conclude that in postmenopausal women, theoretical calculations are valid for the determination of fT and fE<sub>2</sub> concentrations and can give reliable estimation of cancer risk in epidemiological studies when the total concentrations of T, E<sub>2</sub>, and SHBG are measured accurately.**

### Introduction

Epidemiological studies have shown relationships of breast and endometrial cancers with concentrations of T<sup>2</sup> and E<sub>2</sub> in blood among postmenopausal women (1–3). Both T and E<sub>2</sub> are transported in blood bound to proteins (4, 5), of which the most important are albumin and SHBG. Together, these proteins bind >97% of T and E<sub>2</sub> circulating in blood (6). The percentage of T and E<sub>2</sub> that circulates either free (*i.e.*, unbound to any protein) or bound to albumin is defined as the “bioavailable fraction” because only this fraction can potentially cross cellular membranes and bind to the nuclear steroid receptors (7). The bioavailable fraction is virtually equal to the fraction not linked to SHBG (8) and represents >50% of total E<sub>2</sub> and >30% of total T in normal women and men (9). fT and fE<sub>2</sub> are most directly available to tissues under physiological conditions and generally correlate strongly with concentrations of T and E<sub>2</sub> unbound to SHBG (8).

To clarify the roles of fT and fE<sub>2</sub> (and therefore of their bioavailable fractions) in the development of cancer in postmenopausal women, it is important to have precise and inexpensive methods for their measurement that can be easily applied to large-scale epidemiological studies. Several methods have been set up for the measurement of fT and fE<sub>2</sub> rather than for the measurement of the bioavailable fractions because in normal subjects, the free and bioavailable fractions are very highly correlated (8), and the methods for the measurement of the T and E<sub>2</sub> bioavailable fractions require a large amount of biological sample (10–12). The most common methods for measurement of fT and fE<sub>2</sub> are based on dialysis (13, 14), ultrafiltration (15–18), and gel filtration (19, 20). These methods do not measure the absolute concentrations of fT and fE<sub>2</sub> directly but measure fT and fE<sub>2</sub> as percentages of the total circulating T and E<sub>2</sub> concentrations. Absolute levels of fT and fE<sub>2</sub> are then determined by multiplying the percentage of fT or fE<sub>2</sub> with measurements of total T and E<sub>2</sub>, respectively (13). Gel

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<sup>1</sup> To whom requests for reprints should be addressed, at IARC, Unit of Nutrition and Cancer, 150 Cours Albert Thomas, 69372 Lyon, France. Fax: 33-04-72-73-85-75.

<sup>2</sup> The abbreviations used are: T, testosterone; fT, free testosterone; E<sub>2</sub>, estradiol; fE<sub>2</sub>, free estradiol; SHBG, sex hormone-binding globulin; DHT, dihydrotestosterone; fDHT, free dihydrotestosterone; CI, confidence interval.

filtration has been largely abandoned nowadays because it may change thermodynamic equilibrium conditions during the assay, and because it may strip hormones from binding proteins (21). Dialysis is considered as the reference method for the measurement of fT and fE<sub>2</sub>, but it is slow to perform, technically demanding, laborious and expensive, and requires a relatively large sample volume. Ultrafiltration assays are faster, but they remain technically demanding and cumbersome.

An alternative and simple method is to use theoretical calculations of fT and fE<sub>2</sub> from total plasma concentrations of T, E<sub>2</sub>, and SHBG. The free androgen index, calculated as the quotient  $100 \times T/SHBG$  (where  $T$  = total molar concentration of T, and  $SHBG$  = total molar concentration of SHBG in plasma), has been found to be inaccurate as an index of calculation of fT concentration in men, postmenopausal women, and hyperthyroid subjects (22). However, the validity of more complex theoretical calculations, using mass action models based on concentrations of total hormones in blood and their affinity constants for albumin and SHBG (6, 9, 23, 24), has not been extensively examined.

In this paper, we present the results of a study in which we tested the validity of different theoretical calculations for fT and fE<sub>2</sub> in postmenopausal women by comparison with reference values obtained by equilibrium dialysis plus an in-house radioimmunoassay after chromatographic purification. Values of total T and E<sub>2</sub> for fT and fE<sub>2</sub> calculations were obtained by direct, commercially available radioimmunoassays and by an in-house indirect radioimmunoassay. Furthermore, to test the robustness of the theoretical calculations, we simulated the effects on calculated values of fT and fE<sub>2</sub> that may be induced by changes in steroid hormone concentrations and levels of SHBG and albumin (sensitivity analyses).

## Materials and Methods

**Subjects and Blood Collection.** Serum samples were taken from 20 postmenopausal women who participated in the New York University Women's Health Study, an ongoing prospective cohort study in New York. The 20 study subjects were selected at random from a subset of about 2000 women who had donated blood at least four times during the course of the study. For these women, a pooled serum sample of about 24 ml was made from serum samples obtained on two different occasions, which was then re-aliquoted into 1-ml vials and frozen until the measurement of hormones by radioimmunoassays and dialysis.

For one subject, SHBG concentration was very high [213 nmol/liter, a concentration well above the maximum physiological value considered normal in women (between 17 and 87 nmol/liter, a range established on SHBG measurements by the same radioimmunoassay used in this study on more than 1500 postmenopausal women at the Laboratory for Hormone Assays, Unit of Nutrition and Cancer, IARC, Lyon, France)]. This subject was therefore excluded from the study, following the principle that this type of subject would also have been excluded from an epidemiological study, and 19 subjects thus remained for our final analyses.

**Measurements of Total T, Total E<sub>2</sub>, and SHBG Concentrations.** Concentrations of total T and total E<sub>2</sub> were measured as described in detail previously (25). In brief, total T and total E<sub>2</sub> were measured by an in-house radioimmunoassay after extraction by diethyl ether and chromatographic purification on celite columns (celite method), as well as by commercially available direct radioimmunoassays. For total T, direct radioimmunoassays were obtained from Immunotech (Marseille, France),

Orion (Orion Diagnostica, Espoo, Finland), Cis-Bio International (Gif-sur-Yvette, France), Diagnostic System Laboratories (Webster, TX), DiaSorin (Saluggia, Italy), and Byk-Sangtec Diagnostica (Dietzenbach, Germany); for total E<sub>2</sub>, direct radioimmunoassays were obtained from Immunotech, Cis-Bio International, DSL, DiaSorin, and Bio Source Europe (Nivelles, Belgium).

For SHBG, concentrations were measured by two solid-phase sandwich immunoradiometric assays (Cis-Bio International and DSL). The immunoradiometric assay by Cis-Bio International had been validated previously in our laboratories against a reference method based on total T binding capacity (9, 26). The values of SHBG used for the theoretical calculations and for the sensitivity analyses were those obtained by the Cis-Bio International immunoassay.

Measurements by the celite method were done at the Central Laboratory for Biochemistry, Hôpital Neuro-cardiologique (Lyon, France), whereas the direct assays were all performed at the Laboratory for Hormone Assays, Unit of Nutrition and Cancer (IARC).

## Measurements of fT and fE<sub>2</sub> by Equilibrium Dialysis.

Measurements of fT and fE<sub>2</sub> by equilibrium dialysis were done as described in detail previously (13). In brief, dialysis was performed at 37°C using dialysis membranes (Union Carbide, Chicago, IL) for the separation of free and bound fractions of hormones. For each subject, 1 ml of 1:5-diluted serum sample was put into the dialysis membrane, and a known amount of tritiated T or E<sub>2</sub> (<sup>3</sup>H]T or [<sup>3</sup>H]E<sub>2</sub>, about 10,000 cpm in 3 ml of phosphate buffer) was added. After dialysis, 650 μl of the solution inside the dialysis casing and 1 ml of the saline outside the casing were extracted with ethyl ether, and the organic fraction was dried under a gentle stream of nitrogen. The dried extracts were redissolved in 750 μl of saline. Five hundred μl of the solution were then added to 3 ml of scintillation liquid, and [<sup>3</sup>H]T or [<sup>3</sup>H]E<sub>2</sub> activities were counted in a liquid scintillation counter with automatic quench correction. The percentage of the free fraction was then calculated as follows:

$$\% \text{ free} = \frac{D}{R} \times \frac{V_r}{V_d} \times 100$$

where  $D$  is the total concentration of free steroid in volume  $V_d$  outside the casing, and  $R$  is the total concentration of steroid in volume  $V_r$  inside the casing (13).

To calculate the absolute concentrations of fT or fE<sub>2</sub>, the percentage free obtained by dialysis was multiplied by the total concentration of T or E<sub>2</sub>, respectively. Total T and total E<sub>2</sub> were measured by radioimmunoassay after organic extraction and chromatographic prepurification on celite columns (celite method), as described in detail previously (25).

All of the measurements by dialysis plus the celite method were performed at the Central Laboratory for Biochemistry, Service de Radio-analyse et Radiopharmacie, Hôpital Neuro-cardiologique.

## Calculations of fT and fE<sub>2</sub> Using Mass Action Equations.

Two different sets of equations based on the mass action law were used for the calculation of fT and fE<sub>2</sub>.

The first set, previously discussed by Vermeulen *et al.* (23) for fT, relies on the assumption that the concentration of fT (or fE<sub>2</sub>) in blood is determined mainly by the interaction between SHBG and albumin, and total T (or total E<sub>2</sub>) through the different affinity constants of the peptides for these steroid hormones, and that other hormones present in blood do not influence this equilibrium much; that is:

$$[fT] = \frac{([T] - (N \times [fT]))}{(K_s T \{ [C_{SHBG}] - [T] + N_1 [fT] \})}$$

$$\cdot [fE_2] = \frac{([E_2] - (N \times [fE_2]))}{(K_s E_2 \{ [C_{SHBG}] - [E_2] + N_2 [fE_2] \})} \quad (A)$$

where  $[T]$  and  $[E_2]$  are total T and total  $E_2$  concentrations, respectively;  $[fT]$  and  $[fE_2]$  are fT and  $fE_2$  concentrations;  $K_s T$  and  $K_s E_2$  are the affinity constants of SHBG for T and  $E_2$ ;  $N_1 = K_a T C_a + 1$ , and  $N_2 = K_a E_2 C_a + 1$ , where  $C_a$  is the albumin concentration (considered as equal to 43 g/liter  $- 6.5 \cdot 10^{-4}$  mol/liter), and  $K_a T$  and  $K_a E_2$  are the affinity constants of albumin for T and  $E_2$ .

The second set of equations, discussed by Södergard *et al.* (6), is based on the assumption that different hormones present in blood compete for the same protein binding sites, which may have different affinity constants for each of these hormones, and that all of the binding sites of each protein are equivalent and independent.

In our study, we considered only the interactions among T,  $E_2$ , and DHT because in postmenopausal women, these are the most important steroids by their relative concentrations and affinity for the binding proteins. It has been demonstrated earlier that other metabolites (*e.g.*, 5-androstene-3 $\beta$ ,17 $\beta$ -diol and androstane-3 $\alpha$ ,17 $\beta$ -diol) influenced fT and  $fE_2$  concentrations only very slightly (23). Therefore, the system of equations that we considered for the calculations of fT, fDHT, and  $fE_2$  was the following:

$$[E_2] = [fE_2] \left( 1 + \frac{K_a E_2 + [C_a]}{1 + K_a E_2 [fE_2] + K_a T [fT] + K_a DHT [fDHT]} + \frac{K_s E_2 \times [C_{SHBG}]}{1 + K_s E_2 [fE_2] + K_s T [fT] + K_s DHT [fDHT]} \right)$$

$$[T] = [fT] \left( 1 + \frac{K_a T \times [C_a]}{1 + K_a E_2 [fE_2] + K_a T [fT] + K_a DHT [fDHT]} + \frac{K_s T \times [C_{SHBG}]}{1 + K_s E_2 [fE_2] + K_s T [fT] + K_s DHT [fDHT]} \right)$$

$$[DHT] = [fDHT]$$

$$\left( 1 + \frac{K_a DHT \times [C_a]}{1 + K_a E_2 [fE_2] + K_a T [fT] + K_a DHT [fDHT]} + \frac{K_s DHT \times [C_{SHBG}]}{1 + K_s E_2 [fE_2] + K_s T [fT] + K_s DHT [fDHT]} \right) \quad (B)$$

where  $[E_2]$ ,  $[T]$ , and  $[DHT]$  are total concentrations of  $E_2$ , T, and DHT, respectively;  $[fE_2]$ ,  $[fT]$ , and  $[fDHT]$  are concentrations of  $fE_2$ , T, and DHT, respectively;  $[C_a]$  is the concentration of albumin;  $[C_{SHBG}]$  is the concentration of SHBG;  $K_a E_2$ ,  $K_a T$ , and  $K_a DHT$  are the affinity constants of albumin for  $E_2$ , T, and DHT; and  $K_s E_2$ ,  $K_s T$ , and  $K_s DHT$  are the affinity constants of SHBG for  $E_2$ , T, and DHT. This is a system of three nonlinear equations with three unknown variables ( $fE_2$ , fT, and fDHT). The values of the affinity constants used in this study were the following:  $K_s T = 1 \times 10^9$  liters/mol,  $K_a T = 4.06 \times 10^4$  liters/mol,  $K_s E_2 = 3.14 \times 10^8$  liters/mol,  $K_a E_2 = 4.21 \times 10^4$  liters/mol,  $K_s DHT = 3 \times 10^9$  liters/mol, and  $K_a DHT = 3.5 \times 10^4$  liters/mol.

We used Maple software version 6 (Waterloo Maple Inc., Waterloo, Canada) to solve this system of equations and to find values for the three unknown variables (fT,  $fE_2$ , and fDHT).

**Sensitivity Analyses of Mass Action Equations.** Sensitivity analyses on the theoretical calculations consisted of simulating the effects of changes in the total concentrations of  $E_2$ , T, DHT, albumin, and SHBG on (calculated) concentrations of fT and  $fE_2$ . Effects were evaluated mainly by comparison of mean measurement values and by the calculation of Pearson's coefficients of correlation between calculated values before and after changes in total hormone values.

**Comparison of Theoretical Calculations with Equilibrium Dialysis Method.** To validate Eq. A and Eq. B for the calculations of fT and  $fE_2$ , the values of fT and  $fE_2$  obtained by these equations for the 19 study subjects were compared with the values obtained by equilibrium dialysis. All analyses were performed on  $\log_{10}$ -transformed variables to approximately normalize their frequency distributions. Statistical analyses included the calculation of geometric means and CIs and Pearson's correlation coefficients.

## Results

### Sensitivity Analyses

**Influence of Competing Steroids and Albumin Levels on Calculated fT and  $fE_2$  Concentrations.** Variations (in the postmenopausal range) of the concentrations of DHT and  $E_2$  had negligible effects on the measurements of fT by Eq. B, leading to a maximum change in mean fT concentrations of only 0.68% compared with the values obtained when using total T concentrations by the celite method. The same effect was observed on calculated  $fE_2$  when varying DHT and T concentrations in the postmenopausal range (maximum change of 0.45%). Furthermore, the relative ranking of the 19 subjects by calculated values of fT and  $fE_2$  also remained virtually unaffected by these variations in total hormone concentrations (Pearson's correlations  $> 0.996$ ). fT and  $fE_2$  could therefore be calculated by the simpler Eq. A with virtually identical results.

The variation in the absolute levels of albumin between 30 and 60 mg/ml for all of the subjects led to a maximum change of 12% in mean fT concentrations and a 22% variation in mean  $fE_2$  levels. Pearson's coefficients of correlation with the reference values obtained by dialysis plus celite method were always  $> 0.97$ . In addition, the attribution of random albumin values between 40 and 50 g/liter to the 19 subjects had very little effect on the relative classification of these women by relative calculated fT and  $fE_2$  values (Pearson's correlations of 0.97 and higher between fT and  $fE_2$  values obtained Eq. A and reference values obtained by dialysis plus celite method). Thus, changes in absolute levels of albumin concentration affected absolute levels of calculated fT and  $fE_2$  but appeared to have very little effect on the relative classification of subjects from low to high values.

**Influence of Different SHBG Measurements on Calculated fT and  $fE_2$  Concentrations.** Mean SHBG values were 52.68 and 134.58 nmol/liter for the Cis-Bio International and DSL assays, respectively, and Pearson's coefficient of correlation between the two SHBG assays was 0.96. Using total T and total  $E_2$  concentrations from the celite method, the mean values of fT and  $fE_2$  calculated by Eq. A were 2.91 and 0.12 pmol/liter, respectively, when SHBG was measured by the DSL assay, instead of 4.70 and 0.16 when the SHBG assay of Cis-Bio International was used. However, correlations were above 0.995 for fT and  $fE_2$  calculated from the two series of SHBG values.

To simulate the effects on fT and  $fE_2$  concentrations when SHBG values do not correlate with the individual's true levels, concentrations obtained by the Cis-Bio International assay were

randomized among the 19 subjects of the study (randomizations and calculations were repeated 100 times). On average, randomized concentrations of SHBG had close-to-zero Pearson's coefficients of correlation with the individual's original ("true") SHBG values (mean  $r = -0.06$ ). Nevertheless, calculated values of  $fE_2$  remained highly correlated with those calculated with the correct SHBG values (mean value of 0.94), as well as with  $fE_2$  values from the dialysis method (mean value of 0.96). However, the correlations for calculated values of  $fT$  dropped with respect to those where correct SHBG values were used (mean  $r = 0.78$ ) or with respect to the values by dialysis plus celite method ( $r = 0.75$ ).

**Comparison between Calculated Values for  $fT$  and  $fE_2$  and Reference Values by Dialysis.** Geometric means and 95% CIs for  $fT$  and  $fE_2$  obtained by the different methods are shown in Table 1. The SHBG values used in the calculations were those obtained by the Cis-Bio International assay (the Cis-Bio International assay was found to have mean values closest to a reference assay based on steroid-binding capacity<sup>3</sup>). Concentrations of  $fT$  and  $fE_2$  calculated by Eq. A were only slightly higher than those obtained by dialysis plus celite method (10% for  $fT$  and 19% for  $fE_2$ ), when also total T and total  $E_2$  concentrations were measured by the celite method, and when SHBG was measured by the Cis-Bio International kit. However, values of  $fT$  and  $fE_2$  calculated from T and  $E_2$  concentrations by different direct radioimmunoassays were systematically higher than the reference measurements by the dialysis plus celite method. These large variations in mean  $fT$  and  $fE_2$  values paralleled equally large variations in mean levels of the absolute concentrations of T and  $E_2$  by the different immunoassay kits [see detailed results in our previous report (25)].

Pearson's coefficients of correlation between the different methods for measurement or calculation of  $fT$  and  $fE_2$  are in Table 2. For both  $fT$  and  $fE_2$ , the highest correlations ( $r > 0.97$ ) between values calculated by Eq. B and dialysis were found when  $fT$  and  $fE_2$  were calculated from total T and  $E_2$  measured by the celite method using the SHBG kit by Cis-Bio International (Fig. 1, *a* and *b*, respectively). However, good correlations with the reference method ( $r > 0.85$ ) were also found when  $fT$  was calculated from total T concentrations by the direct assay (Immunotech) that gave the highest correlation with total T measured by celite method [ $r = 0.86$ ; see our previous report (25)]. A very similar observation was made for  $fE_2$  (Table 2). Assays that correlated best for total  $E_2$  measurements with the celite method (DiaSorin and DSL,  $r > 0.84$ ) gave good correlations for  $fE_2$  compared with the dialysis plus celite method ( $r > 0.80$ ). Conversely, direct assays of total  $E_2$  that correlated poorly with measurements by the celite method (Immunotech;  $r = 0.29$ ) also resulted in poor correlation ( $r = 0.39$ ) for calculated  $fE_2$  with  $fE_2$  measured by the dialysis plus celite method.

## Discussion

We examined the validity of calculated concentrations of  $fT$  and  $fE_2$  in serum samples from postmenopausal women by sensitivity analyses (theoretical simulations) as well as by comparison with measurements obtained by the equilibrium dialysis and celite method.

Our simulations with Eq. B showed that influences (due to competitive binding) of T and DHT on the calculation  $fE_2$  and influences of DHT and  $E_2$  on the calculation of  $fT$  could be

Table 1 Means and CIs (pmol/liter) of T,  $fT$ ,  $E_2$ , and  $fE_2$  obtained by dialysis and by theoretical calculations from total T and  $E_2$  concentrations<sup>a</sup>

	Celite method	Immunotech	DiaSorin	DSL	Orion	Byk <sup>b</sup>	Cis-Bio
Total T	386.7 (284.7–525.3)	393.4 (279.0–554.7)	641.4 (480.6–855.9)	1219.8 (1009.0–1474.6)	1029.1 (774.0–1368.2)	966.0 (693.8–1345.0)	869.8 (638.9–1184.1)
$fT$ by dialysis	4.21 (3.01–5.90)	4.26 (2.84–6.39)	7.29 (4.87–10.93)	13.67 (9.97–18.75)	11.37 (7.73–16.71)	10.86 (7.25–16.26)	9.74 (6.44–14.71)
$fT$ calculated by Eq. A	4.70 (3.42–6.46)	4.76 (3.23–6.99)	8.16 (5.58–11.96)	15.37 (11.55–20.47)	12.78 (8.89–18.35)	12.20 (8.26–18.02)	10.92 (7.45–16.02)
Total $E_2$	6.81 (4.80–9.68)	67.16 (55.40–81.41)	118.05 (103.69–134.41)	36.51 (30.86–43.20)			
$fE_2$ by dialysis	0.12 (0.08–0.18)	1.21 (0.97–1.49)	2.04 (1.73–2.41)	0.65 (0.54–0.80)			
$fE_2$ Eq. A	0.16 (0.11–0.23)	1.57 (1.26–1.96)	2.66 (2.25–3.15)	0.85 (0.69–1.04)			

<sup>a</sup> SHBG concentrations were measured by the Cis-Bio International assay.

<sup>b</sup> Byk, Byk-Sangtec Diagnostica; Cis-Bio, Cis-Bio International.

<sup>3</sup> H. Déchaud, unpublished results.



Table 2 Pearson's coefficients of correlation and CIs between fT and fE<sub>2</sub> measurements by dialysis plus the celite method and by theoretical calculations (by Eq. A)<sup>a</sup>

A. Total T (CI)							
	Celite	Immunotech	DiaSorin	DSL	Orion	Byk <sup>b</sup>	Cis-Bio
Celite	1.00	0.86 (0.74–0.92)	0.76 (0.59–0.87)	0.76 (0.58–0.87)	0.79 (0.63–0.88)	0.78 (0.62–0.88)	0.70 (0.50–0.83)
B. fT calculated from total T obtained by							
	Celite	Immunotech	DiaSorin	DSL	Orion	Byk	Cis-Bio
Dialysis + celite	0.97 (0.92–0.99)	0.89 (0.74–0.96)	0.80 (0.54–0.92)	0.76 (0.46–0.90)	0.89 (0.73–0.96)	0.75 (0.45–0.90)	0.85 (0.65–0.94)
C. Total E <sub>2</sub> (CI)							
	Celite	Immunotech	DiaSorin	DSL			
Celite	1.00	0.29 (–0.0.3–0.55)	0.86 (0.75–0.93)	0.84 (0.72–0.92)			
D. fE <sub>2</sub> calculated from total E <sub>2</sub> obtained by							
	Celite	Immunotech	DiaSorin	DSL			
Dialysis + celite	0.996 (0.99–1.00)	0.39 (–0.05–0.73)	0.89 (0.73–0.96)	0.80 (0.54–0.92)			

<sup>a</sup> SHBG concentrations were measured by the Cis-Bio International assay.

<sup>b</sup> Byk, Byk-Sangtec Diagnostica; Cis-Bio, Cis-Bio International.

ignored, at least for this random sample of postmenopausal women, and that virtually identical values of fT and fE<sub>2</sub> are obtained by the much simpler Eq. A. This result is most likely explained by the fact that, especially in postmenopausal women with normal (*i.e.*, nonpathologic) levels of endogenous steroids and SHBG, SHBG is present in large excess compared with T and E<sub>2</sub>. Thus, there is only limited competition for the binding sites on this protein. Vermeulen *et al.* (23) made similar observations, even for men and premenopausal women (who have higher concentrations of T and E<sub>2</sub>, respectively, than postmenopausal women), and also proposed the reduced Eq. A for the calculation of fT levels for these population groups. A much simpler equation has the advantages of requiring less data (*i.e.*, no need to measure DHT or additional steroids) and of being solvable by the most common computer programs used for statistical analyses in epidemiological studies (SAS; SAS Institute, Cary, NC).

To validate the measures of fT and fE<sub>2</sub> obtained by theoretical calculations from different measurements of total T, E<sub>2</sub>, and SHBG (by direct immunoassays), we compared them with reference measurements obtained by equilibrium dialysis (to determine the ratio of free:total concentrations of T or E<sub>2</sub>) multiplied by a reference measure of total T or E<sub>2</sub> obtained by indirect immunoassay after organic extraction and purification on celite columns. The use of the celite method as a reference for validation of direct assays of total T and E<sub>2</sub> by commercially available kits has been described in detail previously (25). The concentrations of fT and fE<sub>2</sub> in serum samples from postmenopausal women obtained by dialysis plus the celite method were comparable with those published in the literature (23, 27–31). Comparisons with these reference measurements showed very good validity of calculated values for fT and fE<sub>2</sub> whenever measurements of total plasma T and E<sub>2</sub> corresponded well to the values of the celite method. Furthermore, in situations

where mean levels of direct assays of total T or E<sub>2</sub> were substantially higher than mean celite values, but where those assays correlated strongly with values from the celite method, calculated fT and fE<sub>2</sub> values also remained highly correlated with the reference values based on equilibrium dialysis plus celite method but then had higher mean concentrations of fT or fE<sub>2</sub> as well.

Additional analyses showed that whereas SHBG levels are the major determinant of fT or fE<sub>2</sub> levels measured as a percentage of total T and E<sub>2</sub>, absolute concentrations of fT and fE<sub>2</sub> depended more predominantly on concentrations of total T and E<sub>2</sub>. Measurements of total T, obtained by the celite method or by the different direct radioimmunoassays, showed relatively strong correlations with fT values that were calculated or measured by dialysis (Pearson's correlations between 0.88 and 0.97). By contrast, the percentage of fT did not show any clear correlation with levels of total T (Pearson's correlations varied between –0.23 and 0.39, depending on the combination of assays for T and SHBG from which fT was calculated) but correlated strongly and inversely with SHBG measurements ( $r = -0.94$  for SHBG by Cis-Bio International assay). Similar results were obtained for fE<sub>2</sub>, which correlated directly with total E<sub>2</sub> concentrations measured by the celite method or by the different direct immunoassays (Pearson's correlations between 0.86 and 0.996), whereas fE<sub>2</sub> as a percentage of total E<sub>2</sub> correlated strongly and inversely with SHBG values ( $r = -0.95$  for SHBG by Cis-Bio International assay) but very poorly with total E<sub>2</sub> (correlations between 0.11 and 0.36).

Globally, these results indicate that, at least within a random sample of postmenopausal women with SHBG levels within the normal physiological range, variations in levels of albumin and SHBG did not have much influence on the relative ranking of subjects. This limited influence of SHBG and albumin levels was confirmed by our theoretical simulations, which

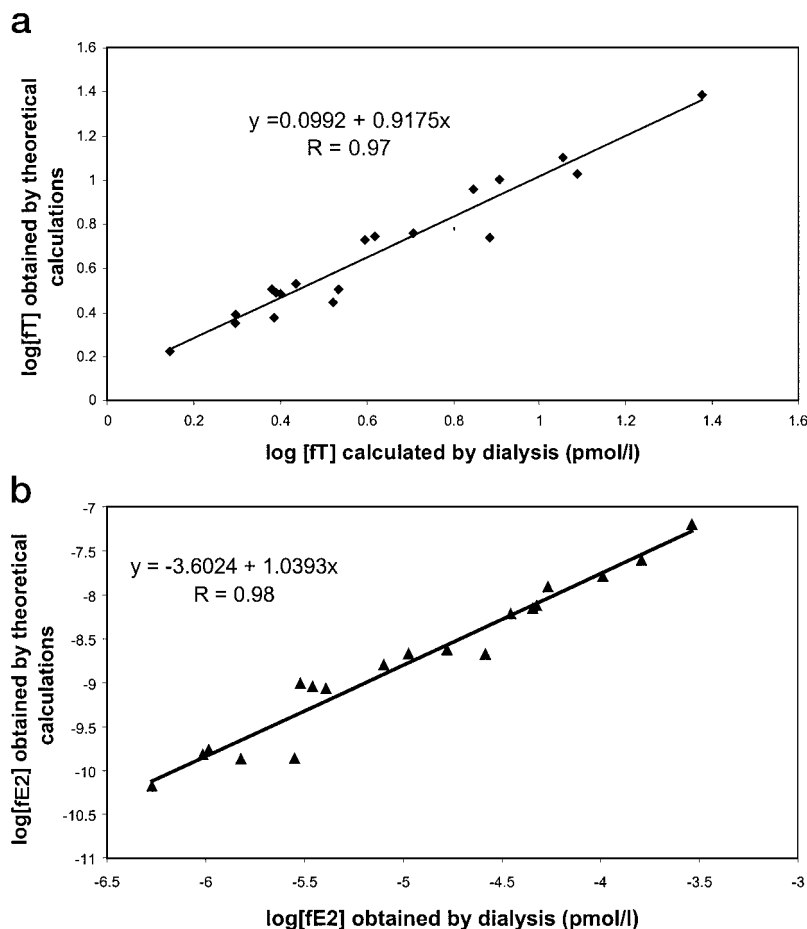


Fig. 1. a and b, relationships between fT and fE<sub>2</sub> (pmol/liter) measured by dialysis and as calculated from SHBG, total T, and total E<sub>2</sub> (total T and E<sub>2</sub> measured by the celite method).

showed that between-subject variations in albumin level, within the normal range of about 40–50 g/liter, had very little influence on the relative classification of subjects by calculated levels of fT and fE<sub>2</sub> and that even changes in albumin concentrations over a broader range (30–60 g/liter) had only modest effects on calculated concentrations of fT and fE<sub>2</sub>. Likewise, between-subject variation in SHBG levels (*e.g.*, randomization of SHBG values of the 19 subjects retained for this study) had only moderate effects on the subjects' classification by relative fT and fE<sub>2</sub> levels. However, our data also showed that correct calibration of the scale of SHBG measurements remains a requirement, if the objective is to obtain accurate mean calculated values of fT and fE<sub>2</sub> (this was illustrated by the difference in mean values for fT and fE<sub>2</sub> calculated from SHBG measurements from the Cis-Bio International or DSL kits).

Although variations in SHBG level had only a modest impact on calculated values of fT and fE<sub>2</sub> in our population sample of normal postmenopausal women, this may not be true for other populations that include subjects with extreme values of SHBG (as in many clinical situations). Indeed, when we considered a population of 90 premenopausal women including cases with both pathologically low and high levels of SHBG (from 5 to 272 nmol/liter) and T (from 5.1 to 104 ng/100 ml), randomization of SHBG levels among the subjects strongly affected the relative ranking of subjects by calculated concentrations of fT (average Pearson's coefficient of correlation of 0.30 with fT values calculated from the correct SHBG concentrations).

In conclusion, theoretical calculations can provide an accurate method for the determination of fE<sub>2</sub> and fT in serum samples from postmenopausal women, provided that the concentrations of total T, total E<sub>2</sub>, and SHBG are measured accurately. Direct radioimmunoassays are methods that best meet the needs of epidemiological studies in terms of speed, cost, and sample volume required. Our study shows that direct assay kits for the measurement of total T and E<sub>2</sub> can be found that allow at least an accurate classification of postmenopausal women by their relative levels of fT and fE<sub>2</sub>, although the exact scaling of measurements, especially for fE<sub>2</sub>, may remain problematic.

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## Validity of Free Testosterone and Free Estradiol Determinations in Serum Samples from Postmenopausal Women by Theoretical Calculations

Sabina Rinaldi, Annabelle Geay, Henri Déchaud, et al.

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