Estradiol-17β Determined in Plasma by Gas Chromatography–Mass Spectrometry with Selected Ion Monitoring of Mixed Silyl Ether–Perfluoroacyl Ester Derivatives and Use of Various Stable-Isotope-Labeled Internal Standards

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A highly specific method is described for measuring estradiol-17β (E₂) in plasma by gas chromatography–mass spectrometry (GC-MS) associated with stable isotope dilution. A mixed derivative, E₂-3-trimethylsilyl ether-17-heptafluorobutyrate (E₂-3-TMS-17-HFB), was found to have excellent analytical properties. The specificity of the derivatization procedure exploits a unique feature of estrogens: the selective exchange of a phenolic perfluorocarbonyl ester for a trialkylsilyl ether. No significant differences in E₂ concentration could be ascribed to the use of ²H₉- or ¹³C-labeled analogs, thus ruling out interferences from possible isotope exchange commonly attributed to deuterated compounds. Precision is closely similar to that for methods in which the more common E₂-3,17-bis(trimethylsilyl) ether and E₂-3,17-bis(hexafluorobutyrate) derivatives are used. Sensitivity and specificity of the mixed 3-TMS-17-HFB derivative allow adequate determinations of E₂, even in plasma from males, in 2-ml samples. Interlaboratory mean concentrations of E₂ obtained by routine immunoassays were consistently higher than the target values estimated by GC-MS, particularly at concentrations <100 pmol/L.

Additional Keyphrases: steroid hormones · derivatization procedures · immunoassays compared

Since the pioneering work of Björkhem et al. (1), Siekmann (2), and others (3–6), validation of routine methods for steroid hormone analysis by reference techniques based on gas chromatography–mass spectrometry (GC-MS) with selected ion monitoring (SIM) has become important in most external quality-assessment schemes (7–9). The reference procedures include solvent extraction, chromatographic fractionation, and chemical derivatization before the instrumental analysis. For unequivocal characterization and reliable quantification of the analyte, the specificity attained during each of these stages should be compatible with that of every other stage. Various efforts to improve the GC-MS determination of estradiol-17β (E₂) have involved efficient liquid-chromatographic separation (10) and solid-phase immunoadsorption combined with high-resolution mass spectrometry (11). Derivatization of E₂ to E₂-3,17-(TMS)₂ has been the most common approach (11–13), but the mass increment provided by trimethylsilylation is rather low; hence, E₂-3,17-(HFB)₂ has been advocated as a better choice for SIM at the higher nominal mass (m/z 664) of the molecular ion (14).

Criteria for the selection of isotopically labeled internal standards have been summarized in a recent review (6). These authors noted that the suspicion most often encountered is that there may have been isotope exchange during the analytical procedure. Therefore, special care should be taken with deuterium-labeled estrogens. Although these are the most easily prepared analogs (15), they present a potential risk for replacement of deuterium by protium.

Here I report the use of novel mixed silyl ether–perfluoroacyl ester derivatives (16) and demonstrate the analytical equivalency of some ²H₉- and ¹³C-labeled internal standards in the development of a reference procedure for the quantification of E₂ in plasma by GC-MS. I also used this method to determine E₂ concentrations in sera distributed by ProBioQual, an external quality-control organization, and compared the results with the assigned target values.

Materials and Methods

Reagents

E₂, a purified preparation from Tenovus Institute, Cardiff, U.K., has been used as a primary reference material in a collaborative project issued by Community Bureau of Reference, Commission of the European Communities, Brussels, Belgium. E₂-¹³C was obtained from CEA Labelled Compounds, Gif-sur-Yvette, France; the ratio of unlabeled to labeled species was 2.6%. E₂-δ₁-δ₂ were synthesized and the isotope composition was determined as described previously (15). Heptafluorobutyric anhydride came from Pierce Chemical Co., Rockford, IL; N,O-bis(trimethylsilyl)- trifluoroacetamide, trimethylchlorosilane, t-butyldimethylsilylchloride, and imidazole were from Fluka, Buchs, Switzerland. The derivatization solvents, acetonitrile and pyridine (E. Merck, Darmstadt, F.R.G.), were made anhydrous by refluxing on phosphorus pentoxide and potassium hydroxide, respectively, then fractionally distilled. Chromatography solvents were of analytical grade and were used without further purification.

Glassware

Volumetric flasks, pipettes, and 25- and 50-µL syringes, used for the addition of internal standards, were calibrated by weighing water samples. Corrections were made for variable densities and for losses due to evaporation.

Apparatus

Gas chromatography–mass spectrometry. GC analysis was performed on a fused-silica column (25 m × 0.32 mm) coated
with OV-73 stationary phase (film thickness 0.15 \( \mu \)m; 85 000 theoretical plates calculated on the \( E_2 \)-3,17-(TMS)\(_2\) peak, with a capacity factor of 5.2) and fitted to an all-glass solid injector heated at 250 °C. The column was operated isothermally at temperatures between 210 and 230 °C with helium as a carrier gas at an average linear velocity of 30 cm/s, and connected directly to the ion source, heated at 180 °C. A quadrupole mass spectrometer (Model 10.10B; Nermag, Rueil-Malmaison, France) was used with normal-source, mass-filter, and electron-multiplier settings: electron impact mode with 70 eV energy of the ionizing electrons at 200 \( \mu \)A filament current, resolution at about 600, and multiplier high voltage at 2.2 kV. Mass calibration was done with perfluorotributylamine. Signals corresponding to the ions selected for quantification were integrated during 200 ms. Data storage and retrieval were done by a PDP Micro-11 Computer (Digital Equipment, Maynard, MA). Peak areas were measured manually with a cross-hair cursor.

Procedures

Sample preparation and extraction. Plasma was obtained either from a local blood bank or, for the low-\( E_2 \)-concentration pool, from male volunteers in the laboratory. The high-concentration pools were prepared by supplementation with 200 ng of \( E_2 \) per liter. Stock solutions of \( E_2 \) analogs, used as internal standards, were prepared in ethanol at a concentration of 20.030 mg/L and the working solutions, in water/ethanol (7/3, by vol), contained 10.136 and 5.068 \( \mu \)g/L. To samples of 0.859 mL for the high concentrations, 1.010 mL for the medium concentration, and 2.222 mL for the low concentration, I added one of the three tested labeled analogs to produce a nearly 1:1 ratio of analyte vs internal standard. Identical volumes of internal standard solutions, measured with calibrated syringes, were added to samples and to standards: 33.3 \( \mu \)L for the high-concentration samples, 12.1 \( \mu \)L for the others. In each run, a sample was analyzed without the addition of labeled analog; this allowed checking for lack of interference on the mass scale of the internal standard. Samples were equilibrated overnight at 4 °C, then extracted with five volumes of dichloromethane. The solvent was evaporated under nitrogen at 50 °C. Lyophilized control sera for external quality-assessment were analyzed similarly.

Liquid chromatography (LC). The dried residue was dissolved in 100 \( \mu \)L of the LC eluent (dichloromethane/methanol/acetic acid, 95/5/1, by vol) and applied to the top of a 120 \( \times \) 4 mm column packed with Sephadex LH-20 (Pharmacia, Uppsala, Sweden) that had been swollen in LC eluent. The first 2.5 mL was discarded and \( E_2 \) was eluted in the next 2 mL. I checked the elution profile each time a new batch of eluent or support was used, using an equimolar mixture of \( E_2 \) and \( E_2 \)-d\(_4\). Elute fractions, 0.5 mL, were collected, evaporated, and the ion abundance ratio (m/z 416/420) was measured after formation of the TMS derivative. No significant separation of the two isotopic species was apparent with this type of low-resolution LC; i.e., in the elution volume of the \( E_2 \), the fractions had similar ion abundance ratios.

Derivatizations

To prepare \( E_2 \)-3-TMS-HFB, I dissolved plasma extracts or standards in 100 \( \mu \)L of anhydrous acetonitrile, added 10 \( \mu \)L of HFB anhydride, and incubated the mixture at room temperature for at least 1 h. After evaporating the reaction mixture under nitrogen at 50 °C, I dissolved the residue in 80 \( \mu \)L of methanol containing pyridine (100 mL/L). After the mixture had reacted for 10 min at room temperature, I evaporated it to dryness, taking special care to remove all traces of methanol. The residue was dissolved in 75 \( \mu \)L of pyridine and 20 \( \mu \)L of \( N,O \)-bis(trimethylsilyl)trifluoroacetamide containing trimethylchlorosilane (50 mL/L). Five minutes later I evaporated the final reaction mixture and dissolved the residual derivatized estrogen in 20 \( \mu \)L of \( n \)-heptane.

To prepare \( E_2 \)-3-TBDMS-17-HFB, I first converted samples and standards to the HFB ester form and dissolved the residue in 80 \( \mu \)L of a solution containing 30 mg of imidazole and 30 mg of \( t \)-butyldimethylsilylchloride in 1 mL of acetonitrile. The mixture was left for 1 h at room temperature and the resulting mixed derivative was extracted into 0.7 mL of \( n \)-hexane after addition of six drops of water. The aqueous phase was discarded, the organic phase was transferred into a dry tube, and the solvent was evaporated. The residue was dissolved in 20 \( \mu \)L of \( n \)-heptane. \( E_2 \)-3,17-(TMS)\(_2\) was prepared with the same trimethylsilylation procedure as that used to form the corresponding mixed derivative.

Analytical recovery. Stable isotope dilution was used for determination of analytical recoveries. Identical samples of one of the high-concentration pools were supplemented with \( E_2 \)-d\(_4\), 300 ng/L, either before extraction and LC, or after these steps; the samples were then derivatized. Measurement of the respective ion abundance ratios, \( d_i/d_0 \) (before extraction) and \( d'_i/d'_0 \) (after LC), yielded a percentage of recovery, calculated as 100 \( \times \) (\( d_i/d_0 \))/(\( d'_i/d'_0 \)), of 71% (SD 2%, n = 4).

Calibration and calculation procedures. Two milligrams of primary standard was weighed on an electronic balance (Micro 4503; Sartorius, Göttingen, F.R.G.) with a precision of 1 \( \mu \)g and dissolved in ethanol to give a concentration of 20.021 mg/L. By a single-step dilution, I prepared two working solutions with concentrations of 6.708 and 3.354 \( \mu \)g/L. In each series, three standard mixtures were analyzed in triplicate: For high-concentration analyses, they contained respectively 224.1, 335.0, and 447.3 pg of \( E_2 \) with 337 pg of internal standard; for the medium- and low-concentration analyses, they contained respectively 81.2, 120.5, and 162.1 pg of \( E_2 \) with 122 pg of internal standard. The standards were evaporated to dryness, derivatized, and dissolved in 20 \( \mu \)L of \( n \)-heptane. Aliquots of 6 \( \mu \)L, both for standards and for plasma samples, were applied to the top of the injection needle. Concentrations of \( E_2 \) were calculated according to Siekmann and Breuer (17). The consistency of the theoretical calibration curves, where the amount of primary standard (\( E_2 \)) was plotted against the ratio (\( y \)) of unlabeled to labeled \( E_2 \), was validated by a positive or zero intercept with the \( y \)-axis.

Results

Gas Chromatography–Mass Spectrometry

The principal characteristics of silyl ether–HFB derivatives of \( E_2 \) are mentioned in Table 1. Of these various mixed derivatives, \( E_2 \)-3-TMS-17-HFB was found particularly adequate on the basis of the mass spectrum shown in Figure 1. The SIM responses of these derivatives are similar to those of \( E_2 \)-3,17(TMS)\(_2\) taken as a reference, but much higher than our detector response of \( E_2 \)-3,17-(HFB)\(_2\), which, nevertheless, has been used successfully for quantification by Siekmann (14). With our instrumentation, the alkali-sensi-
Table 1. GC-MS Characteristics of Estradiol-17β Derivatives Used for Quantification

<table>
<thead>
<tr>
<th>E2 derivative</th>
<th>Retention index*</th>
<th>Principal ion</th>
<th>Relative response^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,17-(TMS)2</td>
<td>2693 M+ 416</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>3,17-(HFB)2</td>
<td>2466 M+ 664</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>3-TMS-17-HFB</td>
<td>2593 M+ 540</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>3-TBDMS-17-HFB</td>
<td>2849 M+ 582</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>[M+-H2+C8H5]+</td>
<td>525</td>
<td>1.00</td>
<td></td>
</tr>
</tbody>
</table>

* Determined with OV-73 stationary phase at 240 °C with hydrogen carrier gas. ^b Detection by SIM.

Table 2. Estradiol-17β Concentrations in a Single Plasma Pool, as Measured with Three Different Internal Standards and with the 3-TMS-17-HFB Derivative

<table>
<thead>
<tr>
<th>Internal standard</th>
<th>Run</th>
<th>Replicates</th>
<th>E2, ng/L</th>
<th>Total variance, ng/L</th>
<th>CV, %</th>
<th>Within-run</th>
<th>Between-run</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p</td>
<td>n</td>
<td>Mean</td>
<td>SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2-d3</td>
<td>1</td>
<td>6</td>
<td>367.5</td>
<td>4.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7</td>
<td>362.3</td>
<td>7.3</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8</td>
<td>385.9</td>
<td>6.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6</td>
<td>361.2</td>
<td>7.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grand mean</td>
<td></td>
<td></td>
<td>364.3</td>
<td>45.0</td>
<td>1.84</td>
<td>43.8</td>
<td>54.6</td>
</tr>
<tr>
<td>E2-d4</td>
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<td>8</td>
<td>357.6</td>
<td>8.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7</td>
<td>365.4</td>
<td>9.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6</td>
<td>390.2</td>
<td>8.6</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9</td>
<td>364.6</td>
<td>7.4</td>
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<tr>
<td>Grand mean</td>
<td></td>
<td></td>
<td>362.0</td>
<td>76.1</td>
<td>2.41</td>
<td>72.7</td>
<td>105.3</td>
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<td>E2-13C</td>
<td>1</td>
<td>8</td>
<td>357.2</td>
<td>9.4</td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>2</td>
<td>8</td>
<td>365.4</td>
<td>8.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7</td>
<td>358.7</td>
<td>7.8</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9</td>
<td>360.4</td>
<td>11.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grand mean</td>
<td></td>
<td></td>
<td>360.5</td>
<td>100.4</td>
<td>2.78</td>
<td>89.3</td>
<td>100.7</td>
</tr>
</tbody>
</table>

*Total variance of pooled data from each of the four runs.
^b F-test with p = 1 degrees of freedom for between-run variance and n = p degrees of freedom for within-run variance; the values of F indicate that differences between the means of each run are nonsignificant (P < 0.05).

Analytical Data

With various labeled analogs as internal standards. Normal distribution of results obtained in four runs with either one of three different labeled E2 analogs and with E2-3-TMS-17-HFB as single derivative was first established by the Kolmogorov-Smirnov test. Analysis of variance was then used to evaluate precision and an F-test indicated nonsignificant differences for within- and between-run variances (Table 2). Data obtained with each of the labeled analogs were consistent; I pooled them to provide grand mean values, which differed insignificantly by Student's t-test.

With various GC derivatives. Another pool was constituted in which E2 was analyzed as three different GC derivatives and with E2-d3 as internal standard. Precision data are given in Table 3, where differences of within- and between-run variances were analyzed by F-test and found to be nonsignificant. Means were compared by t-test; the difference between the results obtained with the derivatives 3,17-(TMS)2 and 3-TBDMS-17-HFB (at m/z 582) was significant (P < 0.01).

Accuracy and precision at medium and low E2 concentrations. Accuracy was evaluated by standard additions (40-160 ng/L range) of E2 to 2-mL samples of a pooled plasma from men, analyzed as the 3-TMS-17-HFB derivative and with E2-d3 as internal standard. Linear regression of found (y) on added (x) E2 gave the equation y = 1.01 (± 0.03)x + 14.2 (± 1.2), where the slope is insignificantly different from 1. Precision data at the other E2 concentrations, determined with the 3-TMS-17-HFB derivative and with E2-d3 as the internal standard, were as follows: at medium concentration, mean, 101.7 ng/L; CV, 3.89%; n = 23, and at low concentration, mean, 14.1 ng/L; CV, 7.09%; n = 24. Figure 2 shows results of analyses of 1-mL aliquots of the medium-concentration pool. It illustrates the sensitivity and the specificity of the analytical procedure, and it demonstrates the absence of interference on the mass scale of the internal standard and thus rules out, at least with this matrix, the possibility of a negative bias.

Analysis of pools for external quality assessment. Aliquots of three serum pools used in the external quality-assessment scheme were analyzed with E2-d3 as internal standard and
3-TMS-17-HFB as derivative. The GC-MS results were lower than the "consensus" mean concentration value calculated from results obtained by different immunoassays performed in various laboratories (Figure 3). Linear regression of the consensus mean value ($y$) on GC-MS data ($x$) gave the equation $y = 0.999x + 65.9 \text{ pmol/L}$.

### Discussion

Most quantitative GC-MS work on E2 has been performed with the 3,17-(TMS)$_2$ derivative, which unfortunately has a rather low nominal mass for its molecular ion. In contrast, the E2-3,17-(HFB)$_2$ derivative has an abundant molecular ion at m/z 664, but the facile hydrolysis of the phenolic perfluoroacyl esters under weakly basic conditions in the vapor phase constitutes a drawback. Thus, the mixed 3-TMS-17-HFB derivative, which is a compromise between the two former derivatives, was proven to have excellent GC and MS properties. Moreover, on a theoretical basis, specificity should be improved, because the selective exchange of a phenolic perfluoroacyl ester for a silyl ether is, among steroids, an exclusive feature of estrogens. It should be stressed, however, that the specificity of the GC-MS is the result of optimization at all the stages of the analytical procedure: extraction, preliminary purification, derivatization, and the resolution capacity of the capillary GC column and of the mass spectrometer.

Quantitative determinations of E2 with 3,17-(TMS)$_2$ and 3-TBDMS-17-HFB derivatives were found to be less precise than with the corresponding 3-TMS-17-HFB derivative (Table 3). This may be ascribed to lack of sensitivity at m/z 582 and to matrix interferences at m/z 525 and 416.

Stable-isotope-labeled analogs are generally considered, aside from the analyte itself, to be the "best possible" internal standards for quantitative GC-MS: their physico-chemical properties are closest to those of the analyte and thus allow for adequate correction of procedural losses. Deuterium-labeled estrogens are easily prepared with high isotopic enrichments (15) and have therefore often been used for quantitative GC-MS (19, 20). However, some systematic errors in the measurement of ion abundance ratios of unlabeled vs labeled compound may be attributed to some deficiencies of the latter, such as exchange of labile isotopes, primary and (or) secondary isotope effects (21), chromatographic isotope effect (22, 23) leading to shorter retention times for deuterated analogs in high-resolution gas-liquid chromatography, and isotope effect on mass fragmentation. Among these drawbacks, deuterium exchange for protium during the analytical procedure is the most often suspected. To evaluate the overall effect of these possible sources of error on the final result of plasma E2 determinations, I did analyses on the same plasma pool, using two different $^{2}$H-labeled analogs and a $^{13}$C-labeled E2.

If some exchange of deuterium for protium occurs, either during the equilibration with the plasma sample or in the preliminary purification stage, then there should be a positive bias for assays performed with $^{2}$H-labeled analogs in comparison with those in which $^{13}$C-labeled analog was used. Deuterium exchange during derivatization and GC analysis would influence the final result to a lesser extent, because both sample and standard would be affected simi-

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**Table 3. Estradiol-17β Concentrations Measured In a Single Plasma Pool, with Three Different GC Derivatives and with E$_2$-d$_3$ as the Internal Standard**

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Nominal m/z of quantified ions</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-TMS-17-HFB</td>
<td>540</td>
<td>344.1</td>
<td>5.9</td>
<td>1.71</td>
</tr>
<tr>
<td>3,17-(TMS)$_2$</td>
<td>416</td>
<td>337.6</td>
<td>11.8</td>
<td>3.50</td>
</tr>
<tr>
<td>3-TBDMS-17-HFB</td>
<td>582</td>
<td>351.8</td>
<td>13.5</td>
<td>3.84</td>
</tr>
<tr>
<td></td>
<td>525</td>
<td>345.7</td>
<td>12.8</td>
<td>3.70</td>
</tr>
</tbody>
</table>

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**Fig. 2.** (A) Analysis of a 1-mL plasma extract with an estradiol-17β content of approximately 100 pg and with a similar amount of E$_2$-d$_3$ (deuterated analog) added as internal standard; (B) analysis of an analogous extract without addition of E$_2$-d$_3$.

**Fig. 3.** Distribution of estradiol-17β concentrations in three serum pools as measured by immunoassay techniques. Data reported by laboratories participating in the ProBioQuaL quality-assessment scheme ($\gamma = \text{group mean}$). The GC-MS results ($\gamma$) are the means of triplicate determinations with E$_2$-3-TMS-17-HFB as derivative and E$_2$-d$_3$ as internal standard.
The data in Table 2 indicate that the analytical behavior of the various internal standards is similar.

The precision attained in the assays with $E_2-d_3$ as internal standard was optimal and may be attributed to the difference of 3 mass units between labeled and native $E_2$, which decreases natural-isotope contributions at the masses of the selected ions and allows linear extrapolation between the bracketing standards (24). Moreover, the retention-time difference between the 3-TMS-17-HFB derivative of $E_2$ and $E_2-d_3$ is less (2 s difference for a retention time of approximately 6 min) than with the corresponding $E_2-d_4$ analog. This makes the respective GC peaks sufficiently coincident and avoids substantial differences in adsorption between the two isotopic species.

Concerning the calibration procedure (17), which is based on linear extrapolation among three standards (molar ratios of unlabeled to labeled $E_2$ were 0.7, 1.0, and 1.3), it should be emphasized that the exact amount of added internal standard does not have to be measured accurately by weighing and dilution if this amount can be calculated and if identical additions of internal standard are made to samples and standards. Thus a source of error in the comparison of various labeled $E_2$ analogs was eliminated.

Precision data obtained with the 3-TMS-17-HFB derivative at different plasma concentrations of $E_2$ are similar to those reported for $E_2$-3,17-(HFB)$_2$ (14). Accuracy, however, may be expected to be better with the more-specific mixed derivative. The "true value" for an endogenous hormone concentration, however, cannot be proved definitively, and the technique of standard additions gives only an indication of the analytical recovery. The collaborative certifications of endogenous steroid concentrations, such as those organized under the auspices of the Community Bureau of Reference (25), in which several independent laboratories analyze the same serum pool according to their well-established GC-MS method, constitute a major improvement towards a better evaluation of accuracy.

The agreement between target values provided by this reference method and mean values calculated from routine analyses performed by different laboratories was fairly good, because the positive bias of the "consensus mean" vs the GC-MS value was within 20% deviation, down to the 300 pmol/L level. At lower $E_2$ concentrations this discrepancy increases (Figure 3, lower panel) and may often be attributed to a higher dispersion of immunoassay results. It is at such low $E_2$ concentrations that a validated reference GC-MS method has the highest potential interest.

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References