Influence of Hemoglobin Variants and Derivatives on Glycohemoglobin Determinations, as Investigated by 102 Laboratories Using 16 Methods

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Influences of hemoglobin (Hb) variants (HbSS, HbCC, β-thalassemia, HbAE, HbAS, HbAC, hereditary persistent HbF) and Hb derivatives (carbamylated- and acetylated-HbS, Schiff base, and those formed in stored blood) on results of glyco-Hb assays by 102 laboratories using 16 different methods were investigated. Affinity chromatography shows deviating results only with homozygous Hb S and C. Correct interpretation of results from patients with decreased erythrocyte half-lives requires previous knowledge on this condition. Measurements of HbA1c by HPLC and electrophoresis are obviously unsuitable for homozygous hemoglobinopathies; for heterozygous hemoglobinopathies and Hb synthesis variants, HbA1c should be expressed as percentage of HbA2 + HbA1c. Abnormal Hbs are usually recognized; both carbamylated- and acetylated-Hbs interfere and Schiff base must be eliminated. Except for stored blood, all Hb variants and derivatives gave erroneous results with disposable ion-exchange columns. Dako’s immunosassay is not affected by Hb derivatives; glycated Hb variants are not recognized as glyco-Hb and percentages are consequently too low. Glyco-Hb by the immunosassay of Bayer (performed by one laboratory) is not affected by Hb variants and derivatives.

Indexing Terms: variation, source of · intermethod comparison

The glycohemoglobin (glyco-Hb) percentage reflects blood glucose concentrations of the previous 2–3 months. It is therefore widely accepted as a valuable indicator for long-term diabetic control (1).

Structural variants of hemoglobin (Hb) contain genetically determined changes in the primary structures of the α-, β-, γ-, and δ-chains, from which HbA0 (α2β2), HbF0 (α2γ2), and HbA2 (α2δ2) are assembled. HbS (β6 glu→val), HbC (β6 glu→lyv), and HbE (β6 glu→lyv), all containing point mutations in the β-chain, are among the most widely occurring Hb structural variants (2). Hb synthesis variants arise from genetically determined changes in the capacity to synthesize Hb chains. β-Thalassemia and hereditary persistent HbF (HPHF), both caused by impaired production of β-chains, arise from these conditions (3). Hb derivatives derive from posttranslational modification of Hb. Examples are those resulting from reactions with glucose (glyco-Hb and the Schiff base precursor, also named pre-HbA1c), urea (carbamylated-Hb), acetyl-CoA or acetylsalicylic acid (acylated-Hb), and storage (4).

Results from glyco-Hb assays by currently used techniques, as well as their interpretation, are differently affected by Hb variants and Hb derivatives (4). The presence of Hb variants and Hb derivatives other than HbA1c (β-chain N-terminal glycated HbA0) and e-N-lysine glycated HbA0 may cause analytical interference. Increased Hb turnover, and consequently decreased exposure time of Hb to circulating glucose concentrations, causes low glyco-Hb percentages. Such conditions may lead to erroneous interpretation, when comparisons are made with reference data obtained from healthy controls.

The aim of this study was to investigate the influence of some Hb structural variants (HbS, HbC, HbE), Hb synthesis variants (β-thalassemia, HPHF), and Hb derivatives (carbamylated-Hb, acetylated-Hb, Schiff base, and those present in stored blood) on the outcome and interpretation of glyco-Hb assays in an external quality-control program in The Netherlands. Assays were performed by 102 laboratories that used 16 different glyco-Hb methods and their own reference ranges.

Materials and Methods

Samples

Lyophilized samples were distributed by mail at ambient temperatures. The participants were instructed to store the vials at 4 °C in the dark and to reconstitute by
adding the method-specific appropriate volume of sample preparation reagent before analysis (5).

**Hb variants.** EDTA-anticoagulated blood was obtained from apparently healthy adults with HbAA, HbAS, HbAC, HbCC, and HPHF, a diabetic adult with HbAE, and a non-diabetic patient with HbSS. β-Thalassemia was simulated by adding one volume of EDTA-blood from a newborn with HbAA to 30 volumes of an EDTA-blood sample from a healthy adult with HbAA. Erythrocytes were washed, hemolyzed, and lyophilized in vials, as previously described (5). The percentages of Hbs in the samples (as determined by a Diamat HPLC system; ref. 6) are given in Table 1.

The study was approved by the local medical ethical committee and was in accordance with the Helsinki Declaration of 1975, as revised in 1983.

**Hb derivatives.** EDTA-anticoagulated blood from the apparently healthy man with HbAA (see above) was used. This subject did not use any medication. Concentrations of blood glucose and serum urea were 4.5 and 5.4 mmol/L, respectively.

EDTA-blood was divided into five portions and processed as follows:

- Control Hb; erythrocytes were washed, hemolyzed, and lyophilized in vials, as previously described (5).
- Carbamylated-Hb; erythrocytes were washed and hemolyzed, followed by synthesis of carbamylated-Hb (7) and lyophilization in vials (5). Formation of carbamylated-Hb was confirmed by J. Barron and E. Carr, using their previously described HPLC method (8).
- Acetylated-Hb; erythrocytes were washed, hemolyzed, followed by synthesis of acetylated-Hb (7) and lyophilization in vials (5). Formation of acetylated-Hb was confirmed by U. Turpeinen, using her previously described method by HPLC (9).
- Stored blood; EDTA-blood was incubated for 7 days at 37°C in the dark. The erythrocytes were washed, hemolyzed, and lyophilized in vials, as previously described (5).
- Schiff base; to prevent mixing of Hb and glucose before reconstitution of lyophilized samples, we placed three frozen layers on top of each other before lyophilization. First, washed erythrocytes were hemolyzed and 250-μL aliquots, containing 4 mmol of Hb (as Fe) per liter, were frozen at −84°C in vials. The second layer consisted of 250 μL of water, which was subsequently frozen at −84°C. The third layer was 250 μL of a 400 mmol/L glucose solution. After lyophilization of the mixture, glucose remained on top of the hemoglobin-cake as a powder.

### Analytical Methods

The contributors quantified glyco-Hb with the following commercially available tests, according to the manufacturer’s instructions:

- **Affinity chromatographic methods.** Modified Pierce test (modification: 10 mL instead of 5 mL of elution wash buffer) and Pierce test (Glycotest II; Pierce, Rockford, IL), Helena test (Quick Column Kit; Helena Laboratories, Gateshead, UK), Iosol test (Glyc-Afin; Iosolab, Akron, OH), and Merck HPLC test (HPLC with Merck Affinity Column 7465; Merck, Darmstadt, Germany).

- **HPLC methods.** Bio-Rad Diamat test (Diamat; Bio-Rad Laboratories, Brea, CA), Bio-Rad Modular-test (designed for Hba1c on a TSK column; Bio-Rad Laboratories), Bio-Rad Column (various HPLC systems equipped with Bio-Rad TSK columns), Pharmacia test (various HPLC systems equipped with Pharmacia Mono-S HR 5/5 columns; Pharmacia-LKB Technology, Uppsala, Sweden), Menarini test (HA 8121; A. Menarini, Florence, Italy), Waters test (Protein-pack Sp 8-HR; Waters Associates Inc., Milford, MA), and Chrompack test (Hb-analyzer; Chrompack Nederland BV, Bergen op Zoom, The Netherlands).

- **Atmospheric pressure ion-exchange chromatography with disposable columns.** Bio-Rad Hba1c test (referred to as disposable ion-exchange columns).

- **Electrophoretic methods.** Beckman test (Diatrac; Beckman Instruments, Brea, CA).

- **Immunochemical methods.** Dako test (Novoclone; Dako, Cambridge, UK), and Bayer test (DCA 2000; Miles, Elk hart, IN).

### Data Evaluation and Statistics

Mean upper and lower glyco-Hb limits for nondiabetic subjects were calculated for each method. For this, both upper and lower reference values, as obtained from participants performing the same method, were averaged. Changes in apparent glyco-Hb percentages in samples with artificially increased Hb derivatives were investigated with the Wilcoxon signed rank test (10). P values <0.05 were considered statistically significant.

### Results

#### Hemoglobin Variants

Figure 1 shows glyco-Hb results for eight samples containing Hb variants, as determined by 102 laboratories using 16 methods. Data are depicted per method and compared with the mean reference ranges reported for the various methods.

#### Homozygous Hba. Most glyco-Hb results of the healthy subject with HbAA fell within indicated refer-
Homozygous HbS. By affinity chromatography, the sample from the patient with HbSS yielded low, but variable, glyco-Hb percentages (Figure 1). Data were
Table 2. Mean Glyco-Hb Percentages in Four Samples Containing Artificially Increased Percentages of Hb-Derivatives

<table>
<thead>
<tr>
<th>Method</th>
<th>No. of labs</th>
<th>Control</th>
<th>Carbamylated-Hb</th>
<th>Acetylated-Hb</th>
<th>Stored blood</th>
<th>Schiff base</th>
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<td>6.0</td>
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Control samples and samples containing artificially increased percentages of Hb derivatives were prepared from a single pool of EDTA-blood from a healthy subject with HbAA.

* Significantly different from control (Wilcoxon rank test, P < 0.05).

b (nm x), number of laboratories reporting “not measurable” (these data were not included in the mean and statistical analyses).

Mean for subset of six laboratories.

Mean for subset of seven laboratories.

30% (Pierce), 55% (Isolab), 65% (Merck), and 80% (Helena) of the respective results reported for the healthy subject with HbAA. With some exceptions, HPLC users were unable to detect any glyco-Hb. Participants who used disposable ion-exchange columns reported low or “not measurable” results. Two participants who used electrophoresis reported “not measurable”; most reported low values; three reported that it could be HbF. Immunoassay results were below the calibration range.

Heterozygous HbC. With affinity chromatography, glyco-Hb percentages for the subject with HbCC were 75% (Pierce), 98% (Isolab), 115% (Merck), and 170% (Helena) of the respective results reported for the healthy subject with HbAA. Most users of HPLC did not detect glyco-Hb. Disposable ion-exchange columns showed low results. With electrophoresis, some laboratories detected no glyco-Hb; others detected low percentages. Immunoassay results were below the zero calibrator for Dako and were within the reference range for Bayer.

β-Thalassemia. Disposable ion-exchange columns showed consistently high glyco-Hb percentages for the non-diabetic subject with simulated β-thalassemia. Results by electrophoresis were variable. All other methods gave glyco-Hb results that were within, or somewhat below, the respective reference ranges.

Heterozygous HbE. Almost consistently increased glyco-Hb percentages in the sample from the diabetic patient with HbAE were found with affinity chromatography, electrophoresis, and immunoassay. Results from HPLC were highly dispersed, ranging from “not measurable” to increased. Disposable ion-exchange columns gave values within the reference range.

Heterozygous HbS. By affinity chromatography, reported glyco-Hb percentages for the subject with HbAS were within the reference ranges. Data from methods by HPLC and electrophoresis were variable, ranging from “not detectable” to increased. Disposable ion-exchange columns gave results within the reference range. Immunoassay percentages were low (Dako) or within the reference range (Bayer).

Heterozygous HbC. Affinity chromatographic results for the subject with HbAC were on the low side of the reference ranges. HPLC and electrophoresis showed glyco-Hb percentages ranging from “not detectable” to within the reference ranges. Disposable ion-exchange columns and immunoassay (Dako) gave decreased values. The result of the (Bayer) immunoassay was within the reference range.

Hereditary persistent HbF. Affinity chromatographic results for glyco-Hb in the sample from the subject with HPHF were on the low side of the reference ranges. HPLC showed values ranging from “not measurable” to within the reference range and a single very high value. Results from disposable ion-exchange columns were invariably high. Data obtained by electrophoresis
ranged from somewhat below the reference range to very high. Immunoassay results were below the reference range (Dako) and normal (Bayer).

Hemoglobin Derivatives

Table 2 shows mean glyco-Hb percentages for a control sample (a healthy subject with HbAA) and samples in which the percentages of carbamylated-Hb, acetylated-Hb, oxidized-Hb, and the Schiff base were artificially increased. The samples containing high percentages of Hb-derivatives other than glyco-Hb were prepared from the control sample by syntheses before lyophilization (carbamylated-Hb, acetylated-Hb, and stored blood) or by spontaneous reaction after reconstitution of the lyophilized sample (Schiff base).

Carbamylated and acetylated hemoglobin. No differences in the percentages for glyco-Hb in the carbamylated and acetylated samples were observed in methods by affinity chromatography and immunoassay. HPLC, disposable ion-exchange columns, and electrophoresis showed increased results.

Stored blood. Neither method showed differences in the percentages for glyco-Hb in the blood sample that was incubated for 7 days at 37°C before lyophilization. Seven HPLC users noted an increased peak at the retention time of HbA1p.

Schiff base. For affinity chromatography, data from the sample with high percentage for Schiff base, caused by a final concentration of 100 μmol of glucose per mole of Hb(Fe) in the reconstituted sample, were usually below those established for the control sample. Results obtained by HPLC and electrophoresis ranged from comparable with the control to increased. No changes were noted by the disposable ion-exchange columns and immunoassay methods.

Discussion

Measurements of glyco-Hb percentages in subjects with Hb variants or high percentages of Hb derivatives may be affected differently in different methods. Decreased exposure time of Hb to circulating glucose concentrations will lead to truly decreased percentages for glyco-Hb, with erroneous interpretation when the condition is not recognized. Compared with subjects with HbAA, patients with HbSS and subjects with HbCC (erythrocyte half-lives: 27–36, 5–10, and 19 days, respectively) (11) will show decreased glyco-Hb percentages that do not relate to mean blood glucose content, unless special reference ranges are used. Specific measurements of HbA1c in homozygous hemoglobinopathies will obviously fail to detect glyco-Hb in these conditions, and will instead necessitate measurements of HbX1c (e.g., Hbs1c and Hbc1c). Expression of HbA1c in terms of total Hb will lead to underestimation of glyco-Hb percentages in conditions characterized by the presence of high percentages of Hb other than HbA1c, such as in subjects with heterozygous hemoglobinopathies (e.g., HbAE, HbAS, and HbAC) or subjects with Hb synthesis variants (e.g., β-thalassemia and HPFH). Measurements of the appropriate derivative/(precursor + derivative) ratios—in general: HbX1c/HbX0 + HbX1c—may overcome these problems. Usefulness of special reference ranges for HbA1c/totol Hb ratios in subjects with heterozygous hemoglobinopathies and β-chain synthesis variants seems questionable, because of the variability of abnormal Hb expression (e.g., the frequent occurrence of concomitant α-thalassemia in subjects of African descent with HbAS (3)) and variability of β-chain expression (e.g., thalassemia major and minor), respectively. Both Hb variants and Hb derivatives may, depending on the technique used, lead to analytical interferences with glyco-Hb.

Table 3 summarizes our findings on the suitability of currently used methods for the assay of glyco-Hb in the presence of Hb variants and Hb derivatives.

Affinity Chromatography

Except for homozygous hemoglobins S and C, for which unexplained highly variable results are seen, affinity chromatographic columns from various manufacturers show similar behaviors. We conclude that the method is not affected by any of the other investigated Hb variants and Hb derivatives. The low glyco-Hb percentages in patients with HbSS and somewhat lower percentages in subjects with HbCC require previous knowledge on these conditions and the use of special reference ranges for correct interpretation. The somewhat lower glyco-Hb percentages obtained from the anal-

Table 3. Suitability of Methods to Determine Glyco-Hb in Samples with Hb Variants and Derivatives

<table>
<thead>
<tr>
<th>Variants</th>
<th>Carbohydrates</th>
<th>Acetate</th>
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<th>Schiff</th>
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<tr>
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<td>U</td>
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<tr>
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<td>Z</td>
<td>Z</td>
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<tr>
<td>AC</td>
<td>Z</td>
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<td>HPHF</td>
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<td>β-Thal</td>
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HPFH, hereditary persistent HbF; β-Thal, β-thalassemia; Carb, carbamylated-Hb; Acet, acetylated-Hb; Stored, stored blood; Schiff, Schiff base (pre-HbA1c).
Z, correct glyco-Hb measured; Y, no interference when Schiff base eliminator is applied; X, interfering substance measured as glyco-Hb; W, interfering substance partially measured as glyco-Hb; V, correct glyco-Hb measured if Hb variant is recognized and mathematical corrections are made before interpretation; U, correct glyco-Hb measured if Hb variant is known and special reference values are used; T, unsuitable to determine glyco-Hb; S, unsuitable to determine glyco-Hb, unless HPLC conditions are changed for measurement of HbS1c/HbC1c.
yses of the Schiff base are most probably caused by decreased glyco-Hb recovery, owing to competition of the abnormally high amounts of glucose added to the sample.

HPLC

Most HPLC methods will recognize abnormal Hbs in the profile. HPLC is obviously unsuitable for measurement of HbA1c in subjects with HbSS and HbCC. Measurements of HbS1c and HbC1c may offer a solution (12), but the outcomes will have to be interpreted by the use of special reference ranges. For subjects with heterozygous hemoglobinopathies and Hb synthesis variants, the method should satisfy three conditions: the Hb variant should be recognized; HbA1c, HbA2, and Hb variants should be separated and reliably quantified; and HbA1c should be expressed as a percentage of HbA0 + HbA1c. Alternatively, for those samples that show Hb variants in the chromatograms, HPLC users can additionally use a method that is not affected by Hb variants, such as affinity chromatography or immunosay.

Carbamylated- and acetylated-Hb coelute with HbA1c, causing falsely increased results. Interference by carbamylated-Hb is a serious problem because it cannot be recognized from the chromatogram. Its increased concentrations in uremic patients may be important (7). Two strategies might help to detect hyperuricaemia: (a) clinicians can be encouraged to identify such patients or (b) the laboratory data system can be programmed to list available urea concentrations along with glyco-Hb results before validation. After recognition of hyperuricaemia, the sample may be reexamined by a method that is not affected by carbamylated-Hb (affinity chromatography, immunosay), or the results may be corrected by subtraction of 0.06% for each 1 mmol/L of blood-urea (7). The Schiff base also coelutes with HbA1c, but its destruction during sample preparation eliminates the problem. Storage of blood causes no artifacts.

Disposable Ion-Exchange Column

Results of HbA1c measurements with disposable columns are grossly comparable with those of HPLC. In contrast to HPLC, the method does not recognize abnormal Hb; consequently, corrections for heterozygous hemoglobinopathies and Hb synthesis variants are impossible. The method is unsuitable for HbA1c analyses in samples with increased HbF. In view of the multitude of imperfections, we believe this method should be replaced.

Electrophoresis

As with HPLC, electrophoresis is obviously unsuitable for measurement of HbA1c in samples from subjects with homozygous hemoglobinopathies. Most electrophoretic methods recognize Hb variants. Because the electrophoretic technique for HbA1c does not separate HbA0 from HbE0 or HbA1c from HbE1c, glyco-Hb was increased for the diabetic patient with HbAE. In our opinion, reexamination of samples that show Hb variants, either by affinity chromatography or immunochromatographic methods, is the best strategy to avoid unreliable results. Alternatively, although less straightforward than with HPLC, HbA1c can be expressed as a percentage of HbA0 + HbA1c. In electrophoresis, carbamylated-Hb is measured as HbA1c, and acetylated-Hb is at best only partially resolved from HbA1c. To avoid misinterpretation, we propose the same procedures as described for HPLC. The Schiff base comigrates with HbA1c, but this can be circumvented by eliminating the base before analysis.

Immunosay

Because of the specificities of the antibodies, the immunochromatographic methods of Dako and Bayer behave differently. The Dako antibody recognizes only HbA1c, this causes responses towards Hb variants similar to those of HPLC, except that this immunosay does not recognize Hb variants and corrections by taking HbA1c/(HbA0 + HbA1c) ratios are impossible. Improbably low percentages of glyco-Hb, especially those below the reference range, are clear indications for the presence of Hb variants. After establishment of the condition by other techniques, Dako users may deal with the Hb variants either by using special reference ranges or by using a method that is not affected. The antibody of the Bayer assay recognizes glyco-Hbs with glucose moieties at the β-chain N-terminal. Consequently, it recognizes the investigated HbX1c. This causes results similar to affinity chromatography, except that e-N-lysine glycated Hb is not measured. For correct interpretation of results for subjects with decreased erythrocyte half-life, the condition should be known beforehand. Because only one laboratory used the Bayer method, the results are less firm than those for other methods. Neither immunochromatographic method is affected by Hb derivatives.

We thank W. V. Martina for supplying us with samples from subjects with HbSS, HbCC, HbAS, HbAC, and HPHF; G. A. van den Berg for the sample from the patient with HbAE; J. Barron and E. Carr for confirmation of carbamylated-Hb synthesis; U. Turpeinen for confirmation of acetylated-Hb synthesis; and all our colleagues for their participation in this study.

References

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Within- and Between-Subject Biological Variations of Folitropin, Lutropin, Testosterone, and Sex-Hormone-Binding Globulin in Men

José Valero-Fonti and Xavier Fuentes-Arderiu

The within-subject and between-subject biological variation of the serum concentrations of folitropin, lutropin, sex-hormone-binding globulin, and testosterone; the ratio between the serum concentrations of testosterone and sex-hormone-binding globulin; and the concentration of testosterone in saliva have been studied in a group of 20 men during 12 months. The between-subject coefficients of variation (CVs) were 36.0% for folitropin, 37.0% for lutropin, 42.7% for sex-hormone-binding globulin, 21.3% for testosterone in serum, 28.8% for testosterone in saliva, and 51.6% for the ratio between serum concentrations of testosterone and sex-hormone-binding globulin. The medians of the within-subject CVs for the respective analyses and ratio were 17.3%, 24.0%, 12.1%, 10.9%, 17.3%, and 9.4%. These data were used to calculate the desirable imprecision, the critical difference for significant change detection, and the index of individuality.

Indexing Terms: variation, source of • analytical goals • critical difference • steroid hormones • saliva

The applications in clinical biochemistry of the data on within-subject biological variation of biochemical quantities are well established (7). These applications include establishment of metrological or analytical goals, evaluation of the significance of a change between two successive results, assessment of the suitability of the population reference values, establishment of the number of specimens to be collected and analyzed for the estimation of the "homeostatic value" of a quantity, and selection between biochemical quantities with the same clinical utility. Here we provide data on within-subject and between-subject biological variation of the serum concentrations of folitropin, lutropin, sex-hormone-binding globulin, and testosterone; the ratio between the serum concentrations of testosterone and sex-hormone-binding globulin; and the concentration of testosterone in saliva in men. To our knowledge, there is only one publication on this topic, in which the biological variation of the serum concentration of folitropin, lutropin, and testosterone were studied over a period of 7 days (2).

Materials and Methods

Subjects and Specimens

The participants were 20 apparently healthy men, ages 26 to 467 years. These volunteers maintained their usual life styles, which involved no strenuous exercise, throughout the studied period; none of them was taking any medication.

During 12 months, at −1-month intervals, venous blood and salivary (unstimulated) specimens were collected between 0800 and 0930 h. Venous blood specimens were drawn, with the volunteers in a sitting position, usually by a single phlebotomist and with minimal stasis, using the Venoject system (Terumo Europe, Leuven, Belgium), with 0.9 × 25 mm needles. Blood and saliva were centrifuged at 1400 × g for 10 min and the resulting serum and salivary specimens were frozen at −80 °C until assayed. Using this protocol, the premetrological variation was considered negligible.

All procedures followed were in accordance with ethical standards of the hospital where the work was done.

Measurements

Concentrations of folitropin and lutropin were measured by fluoroenzymoimmunoassay (Stratus Immunoassay Systems; Baxter Diagnostics Inc, Miami, FL), and concentrations of testosterone and sex-hormone-binding globulin were measured in duplicate by radioimmunoassay (Extraction Testosterone [125I] radioimmunoassay kit, and Sex Hormone Binding Globulin [125I] immunoradiometric assay kit; both from Farmos Diagnostica, Oulunsalo, Finland). All specimens collected in the same month were analyzed within the same run.

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