percentile method suggested by Harrell and Davis estimation. Should be considered for nonparametric reference interval uncertainty also suggests that a sample size of at least 100 exceeding 100. In addition, the high general degree of relevance, e.g., the log-normal distribution, the skew distributions at all studied sample sizes. This was also brought into focus in the present study. The above-mentioned expressions have actually been considered for use in clinical chemistry, but they have not received much attention (12, 13).

Overall, procedure IIB provided the lowest RMSE of percentiles for both the gaussian and the skewed distributions at all studied sample sizes. This was also confirmed for other types of distributions that might be of relevance, e.g., the log-normal distribution, the skew model of Box and Cox, and symmetric distributions with kurtosis deviating from that of the gaussian distribution (11). However, at small sample sizes, the estimated confidence interval has a low coverage. Thus, it is advisable to apply the bootstrap procedure mainly at sample sizes exceeding 100. In addition, the high general degree of uncertainty also suggests that a sample size of at least 100 should be considered for nonparametric reference interval estimation.

The bootstrap procedure is related to the weighted percentile method suggested by Harrell and Davis (14). Percentiles are estimated as a weighted average of all possible percentiles, which may reduce the RMSE by ~10–15% for a sample size of 119 (3, 14).

The present study shows that irrespective of what type of nonparametric procedure is used, nonparametric reference interval estimation at small-to-moderate sample sizes is associated with a large degree of uncertainty. A minimum sample size of 120 for nonparametric reference interval estimation has been suggested previously with reference to the lower limit for specification of the 90% confidence intervals of the percentile limits on basis of the sorted sample values. At this sample size, the width of the 90% confidence interval is likely to be <20% of the length of the reference interval, given a symmetric distribution, but for skewed distributions, the percentage is larger. For sample sizes exceeding 100, the bootstrap procedure, preferably in the IIB version, can be recommended, and the improvement in efficiency is likely to correspond to sample size savings of 10–15%. The bootstrap procedure for reference interval estimation is available in the RefVal program (IB version) distributed by Solberg (15), and in the CBstat program (IB and IIB versions), which is a Windows program distributed (free) by the author (16).

References


Estimation of Serum Apolipoprotein B by a Modified Homogeneous Assay for HDL-Cholesterol, Maureen L. Sampson, Gyorgy Csako, and Alan T. Remaley* (Clinical Pathology Department, Clinical Center, National Institutes of Health, Bldg. 10, Rm. 2C-407, Bethesda, MD 20892-1508; * author for correspondence: fax 301-402-1885, e-mail aremaley@nih.gov)

Serum lipoprotein analysis frequently is used in assessing the risk for coronary artery disease and for monitoring cholesterol-lowering therapy (1, 2). A recent improvement in the analysis of lipoproteins is the development of homogeneous assays for HDL-cholesterol (HDL-C) (3, 4) that are easier to perform because they do not require the

<table>
<thead>
<tr>
<th>Sample size</th>
<th>Gaussian IIS</th>
<th>IIB</th>
<th>Skewed distribution IIS</th>
<th>IIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>9.5</td>
<td>8.6</td>
<td>24.7</td>
<td>21.6</td>
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<td>120</td>
<td>5.9</td>
<td>5.4</td>
<td>15.2</td>
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<tr>
<td>250</td>
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<td>500</td>
<td>3.0</td>
<td>2.8</td>
<td>7.2</td>
<td>6.9</td>
</tr>
</tbody>
</table>

*a For the skewed distribution, the SE refers to the upper percentile estimate.
physical separation of the apolipoprotein B (apoB)-containing lipoproteins by precipitation and centrifugation. The measurement of LDL-cholesterol (LDL-C) and apoB, the principal protein on LDL, is also useful for estimating the risk for cardiovascular risk (1, 2). An isolated increase in apoB in the absence of increased total cholesterol and LDL-C is diagnostic for a risk condition called hyperapoB-lipoproteinemia (5). We describe a simple modification of an antibody-based commercial homogeneous assay for HDL-C (EZ-HDL; Sigma Diagnostics) that in addition to measuring HDL-C, also provides an estimate of the apoB concentration.

In step 1 of the EZ-HDL assay, an anti-apoB antibody is added, which binds to the surface of the apoB-containing lipoproteins. In step 2, the reagents for the enzymatic detection of “accessible” cholesterol are added, which produces an absorbance (600 nm) change in step 2 that is proportional to the concentration of HDL-C. The assay is specific for HDL-C because the anti-apoB antibody sterically blocks cholesterol oxidase from reacting with cholesterol on the apoB-containing lipoproteins. While routinely using the EZ-HDL assay, we often observed, particularly in specimens with high LDL-C, an upward shift in the absorbance at the end of the 4-min antigen-antibody incubation, just before the addition of the reagents in step 2 for the enzymatic detection of cholesterol (Fig. 1A). A similar change in absorbance was observed upon the addition of purified LDL but not HDL (Fig. 1A), which indicates that the increase in turbidity in step 1 is the result of the binding of the anti-apoB antibody to the apoB-containing lipoproteins. Because it is a relatively abundant serum protein, apoB is routinely measured by turbidimetric methods (6) that depend on the physical aggregation of apoB-containing lipoproteins after incubation with an anti-apoB antibody. Taking advantage of this phenomenon, we assessed the turbidity change at 600 nm during the first incubation step of the EZ-HDL assay as a possible measure of serum apoB.

In Fig. 1B, the measurement of apoB by the turbidimetric change in the EZ-HDL assay was compared with a standard nephelometric assay for apoB (Protein Array; Beckman). An endpoint calculation was used in measuring the turbidity of the EZ-HDL assay by subtracting the background absorbance reading at 0.5 s from the absorbance reading at 4 min at the end of step 1. A high apoB (1.43 g/L) and a low apoB (0.51 g/L) serum pool, as measured by the standard assay, were used to calibrate the first step of the EZ-HDL assay. The turbidimetric measurement from the EZ-HDL assay correlated relatively well ($r = 0.92$) with the standard assay for nonlipemic specimens (Fig. 1B, ○). The assay also had an acceptable (7) intraassay CV of 2.9% at 0.59 g/L ($\text{n} = 20$) and an interassay CV of 4.6% at 1.23 g/L ($\text{n} = 10$). Lipemic specimens (triglycerides, 3.05–9.83 mmol/L; total cholesterol, 4.95–7.69 mmol/L), which had a baseline absorbance $>0.016$ (Fig. 1B, ◦), often deviated from the standard assay and typically showed a negative bias. Although this is a limitation of the assay, the ability to identify specimens that are not suitable for analysis by the baseline absorbance should be helpful.

The modified EZ-HDL assay potentially represents a cost-effective procedure for performing lipoprotein analysis because it provides a simultaneous measure of HDL-C and apoB in a single test. The modification of the assay involves calibrating the assay for apoB and monitoring the absorbance during the first incubation step, and it does not require any additional reagents. Unlike HDL-C, apoB often is not measured initially in the screening for hyperlipidemia. The modified EZ-HDL assay may, therefore, provide a convenient way for identifying patients with hyperapoB-lipoproteinemia (5) who might not otherwise be diagnosed. Given the limitations of the assay.

![Fig. 1. Estimation of apoB by the EZ-HDL assay.](image-url)

(A), change in absorbance during step 1 incubation. The absorbance reading at 0.5 s was subtracted from the reading at 4 min for a serum sample (LDL-C, 6.89 mmol/L), purified LDL (LDL-C, 6.1 mmol/L), and purified HDL (HDL-C, 4.45 mmol/L) prepared by ultracentrifugation. (B), comparison by Deming regression of the modified EZ-HDL method and a standard assay (Protein Array) for measuring serum apoB in nonlipemic samples (○; baseline absorbance $<0.018$; $y = 1.12x + 0.15$ g/L; $S_{xy} = 0.09$; $r = 0.92$; $n = 74$) and lipemic samples (◦; baseline absorbance $>0.018$).
in regard to standardization and interference by lipemia, however, it should not be viewed, however, as a replacement for a standard assay of apoB. Any specimen found to have increased apoB by the EZ-HDL assay should be confirmed with a standard apoB test.

References

Latex-enhanced Immunoturbidimetry Allows D-Dimer Determination in Plasma and Serum Samples, Wolfgang Korte* and Walter Riesen (Institute for Clinical Chemistry and Hematology, Kantonsspital, 9007 St. Gallen, Switzerland; * author for correspondence: fax 41-71-494-3900, e-mail Wolfgang.Korte@gd-ikch.sg.ch)

Quantitative D-dimer determination has become routine practice in patients evaluated for the presence of deep venous thrombosis or pulmonary embolism (1–3). D-Dimer concentrations below a certain cutoff specifically defined for each assay [500 μg/L for ELISA and comparable assays (3)] are considered sufficient evidence to exclude a deep venous thrombosis or pulmonary embolism if the pretest probability is low (4). In addition, D-dimer has been shown to be a reliable indicator of coagulation activation in disseminated intravascular coagulation (5) and malignancy (6). More recently, the relevance of the determination of D-dimer in arterial disease was evaluated (7), and it was shown that D-dimer is a very good predictor of recurrent acute coronary syndromes after a first event (8). There is also some indication that the amount of D-dimer generated correlates to some extent with the degree of atherosclerosis (9).

Fully quantitative D-dimer assays and their automation are recent improvements (10), and short turnaround times allow the routine use of such assays. Routinely, plasma is used for the D-dimer assays. Serum is believed not suitable because of the possibility of continued fibrinolytic activity, which (theoretically) could lead to a (falsely) increased D-dimer concentration. Here, we report that latex-enhanced immunoturbidimetric measurement allows the use of serum as a matrix for the measurement of D-dimer concentrations. Samples were from patients who had a D-dimer test (from citrated plasma) ordered as well as available serum obtained during the same blood collection. The samples were selected without conscious bias during a 5-week period. Routine blood samples were collected with the Vacutainer® system (Becton Dickinson). For citrated plasma, blood (3.6 mL) was collected into 0.125 mol/L sodium citrate (0.4 mL). For serum, blood was collected into 10-mL tubes containing polystyrene granules. When the samples arrived in the laboratory, platelet-poor plasma was prepared from the citrated samples by centrifugation (1600g for 10 min at 22°C); serum was obtained by centrifugation (1500g for 6 min at 9°C). D-Dimer concentrations were determined using a latex-enhanced immunoturbidimetric assay (Tinaquant D-dimer on a Hitachi 917 analyzer; Roche Diagnostics). All D-dimer determinations from plasma and serum were performed according to the same routine protocol. D-Dimer concentrations were determined from both materials with three different methods of sample processing: (a) immediately after centrifugation (n = 33); (b) after incubation of the centrifuged original tubes at 4°C for 24 h (i.e., with the cell sediment/clot in place; n = 13); and (c) after incubation of

Table 1. Testing for significant differences between D-dimer concentrations measured in plasma and serum and with three different methods of processing.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Immediate Processing</th>
<th>n</th>
<th>P for Difference*</th>
<th>24 h at 4 ºC</th>
<th>P for Difference*</th>
<th>24 h at 4 ºC</th>
<th>Supernatant, 24 h at 4 ºC</th>
<th>P for Difference*</th>
<th>Immediate Processing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Plasma D-dimer, mg/L</td>
<td>0.81</td>
<td>0.678</td>
<td>13</td>
<td>0.64</td>
<td>0.942</td>
<td>0.68</td>
<td>0.701</td>
<td>0.81</td>
<td></td>
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<tr>
<td>Median Serum D-dimer, mg/L</td>
<td>0.77</td>
<td>0.760</td>
<td>14</td>
<td>0.72</td>
<td>0.846</td>
<td>0.77</td>
<td>0.926</td>
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<tr>
<td>R² for Plasma vs Serum</td>
<td>0.989</td>
<td>0.993</td>
<td>0.974</td>
<td>0.989</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Difference between processing immediately after centrifugation vs processing after incubation of the centrifuged sample in the original tube (with clot and cell sediment present) for 24 h at 4°C.

* Difference between incubation of the centrifuged sample in the original tube (with clot and cell sediment present) for 24 h at 4°C vs processing immediately after centrifugation.