Revised Calibration of the Reflotron Cholesterol Assay Evaluated

G. J. M. Boerma,1 I. van Gorp,1 T. L. Liem,1 B. Leijne,1 J. Belim,2 and C. A. Carstensen2

We evaluated the Boehringer Mannheim (B.M.) Reflotron Total Cholesterol "dry-chemistry" method after its recalibration in 1987. In the literature up to 1986–1987 of a negative bias (up to −10%) in the method prompted a revision of the factory-set calibration of the Reflotron. For this, B.M. prepared a new set of calibrators with 12 different concentrations of cholesterol. We checked in two ways whether accuracy had been achieved: (a) The values assigned to the calibrators by B.M. were checked with the manual Abell–Kendall Reference Method (MAK) performed in an official Reference Center. These were shown to be correct. (b) Concurrently, a direct comparison was made by analyzing 200 fresh samples of human serum. Reflotron cholesterol values obtained for these samples proved to be accurate, meeting the current World Health Organization/Centers for Disease Control criterion of maximum bias ≤ 5%. Orthogonal regression analysis yielded the following correlation: Reflotron = 0.985 MAK + 0.238 mmol/L (y = ax + b). Reflotron mean = 6.26 mmol/L; MAK mean = 6.09 mmol/L. SDa = 0.015 mmol/L; SDb = 0.120 mmol/L, and r = 0.989.

Several chemical analyzers have recently been developed based on dry-chemistry methodology (1), facilitating opportunities for monitoring patients, screening, controlling therapy, and doing emergency clinical testing. Ease of operation, small space requirement, financial benefit for the user, and other advantages have encouraged the use of these analyzers in laboratories, physicians' offices, and hospital clinics (2, 3).

One such analyzer, the "Reflotron System" (Boehringer Mannheim, Mannheim, F.R.G.) (2–5) involves the use of a dip-stick type of reagent carrier. On the strip a 30-μL sample (whole blood, serum, or plasma) must be applied to the 6 × 6 mm2 red protective filter, which retains the cells. The plasma flows into a reagent tab by capillary action, then reacts with chemicals present in various layers of thin filter paper. This process is started when pressure is exerted on the pad as the strip is positioned in the Reflotron. The result appears in 3 min. The Reflotron reagent strips are assembled in such a way as to utilize the following reaction sequence: enzymatic hydrolysis of cholesterol esters with cholesterol esterase, oxidation of total cholesterol with cholesterol oxidase, and concomitant production of hydrogen peroxide, leading to the formation of a colored product, which is quantified by photometry. A reflectance photometer of the type used does not show a linear Lambert–Beer relationship between the detected light intensity and the concentration of colored product.

Calibrators with 12 different concentrations are used to establish the nonlinear calibration curve.

The user calibrates the assay by entering data into the analyzer, specifying the lot-specific calibration curve by means of a magnetic tape attached to the back of each test strip (in some production lots on a separate strip).

Most method comparisons between Reflotron and "wet chemistry" cholesterol procedures published so far include no proof of standardization of the comparison method. Evaluation of accuracy of the Reflotron is then impossible. Other confusion may arise from comparing venous with capillary (finger-stick) blood.

Some publications on analytical aspects of this type of dry-chemistry test (6–13) indicate that Reflotron cholesterol results acquired before 1986–1987 are too low by approximately 10% (6, 9, 11). Recent reports show better accuracy when newer reagents were used (10, 12).

Here we present our assessment of whether Boehringer's newly produced set of serum calibrators and the reprogrammed calibration curve have corrected this negative bias in cholesterol results. We first verified the tentative target values for the new Reflotron calibrator set; then we checked the accuracy of Reflotron Cholesterol by establishing the correlation with the manual Abell–Kendall (MAK) Reference Method (14, 15) as well as with our standardized enzymatic assay (16), using 200 samples of human serum.

Materials and Methods

Reference method (14). The Lipid Reference Laboratory in The Netherlands uses the Centers for Disease Control (CDC) version of the MAK for total cholesterol. Every three months, a direct comparison is made with CDC reference serum pools. A summary of these results is given in Table 1, to demonstrate our comparability with the CDC Lipid Reference Laboratory. Our Reference Laboratory is the European representative in the network of standardized reference laboratories constituting the National Reference System in the U.S.A. This System will assist industries in establishing "certified target values" for several control and calibration materials (15).

Table 1. Accuracy of the Manual Abell–Kendall Reference Method as Assessed at the Netherlands Lipid Reference Center

<table>
<thead>
<tr>
<th>Year</th>
<th>TC*</th>
<th>HDL-C</th>
<th>Rotterdam valuea</th>
<th>TC</th>
<th>HDL-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1983</td>
<td>8.56</td>
<td>0.69</td>
<td>8.61 (0.080)</td>
<td>0.68 (0.020)</td>
<td></td>
</tr>
<tr>
<td>1984</td>
<td>4.20</td>
<td>1.27</td>
<td>4.24 (0.050)</td>
<td>1.29 (0.010)</td>
<td></td>
</tr>
<tr>
<td>1985</td>
<td>5.93</td>
<td>0.91</td>
<td>5.99 (0.019)</td>
<td>0.65 (0.046)</td>
<td></td>
</tr>
<tr>
<td>1986</td>
<td>0.73</td>
<td>1.66</td>
<td>0.72 (0.020)</td>
<td>1.63 (0.011)</td>
<td></td>
</tr>
<tr>
<td>1987</td>
<td>4.20</td>
<td>0.91</td>
<td>4.16 (0.032)</td>
<td>0.89 (0.016)</td>
<td></td>
</tr>
<tr>
<td>1988</td>
<td>7.90</td>
<td>1.66</td>
<td>7.84 (0.026)</td>
<td>1.63 (0.019)</td>
<td></td>
</tr>
<tr>
<td>1989</td>
<td>3.15</td>
<td>0.94</td>
<td>3.15 (0.023)</td>
<td>0.90 (0.015)</td>
<td></td>
</tr>
<tr>
<td>1990</td>
<td>9.04</td>
<td>1.63</td>
<td>9.02 (0.040)</td>
<td>1.63 (0.015)</td>
<td></td>
</tr>
<tr>
<td>1991</td>
<td>8.99</td>
<td>1.66</td>
<td>8.94 (0.023)</td>
<td>1.62 (0.042)</td>
<td></td>
</tr>
<tr>
<td>1992</td>
<td>1.11</td>
<td>1.24</td>
<td>1.10 (0.010)</td>
<td>1.19 (0.014)</td>
<td></td>
</tr>
</tbody>
</table>

*TC, total cholesterol; HDL-C, high-density-lipoprotein cholesterol. aMean (and SD) of nine determinations each, selected randomly from results for CDC serum controls sent to Rotterdam each quarter for the CDC/NHBLI standardization program.
Enzymatic standardized method (16). To study cardiovascular risk in epidemiological studies and trends in risk indicators, we use a cholesterol oxidase–p-aminophenazone (ChOD-PAP) procedure (Monotest Cholesterol high-performance kit no. 236691; lot no. 15648101-92, B.M.). This method is standardized in the Netherlands Cholesterol Standardization Programme (17, 18). A serum calibrator is prepared in-house each year with use of aliquots of human serum from the Department of Clinical Chemistry, which are visually inspected to avoid lipemia, turbidity, hemolysis, and high bilirubin. Pools, 100 mL each, are tested for antibodies to HIV and hepatitis-B. Only lots found negative for both are included in the final pool, which is then filtered, homogenized well, and dispensed into several hundred 2-mL vials. These are stored at −20 °C for up to one year.

Reflotron System. Three Reflotron analyzers were kindly made available by Boehringer Mannheim (through Almere, The Netherlands.) The instruments were used according to the instructions in the manual, and set at 37 °C. The Reflotron Cholesterol does not measure values <2.6 mmol/L.

Reflotron System cholesterol reagents. A recent lot number of Reflotron Cholesterol reagent carriers was used: lot no. 234081/31, id. no. 881937. With this lot a separate tape carrying the calibration data was provided.

Serum control materials. We analyzed several commercial lyophilized control materials on each day of the project: Precinorm L (PFL) id. no. 781827, lot 154757; Precipath U (PFU) id. no. 745162, lot 154315; Precinorm U (PNU) id. no. 745154, lot 154313 (all from Boehringer Mannheim); QAP Chemistry Control level I (QAP), lot 523.02; and LipTrol, lot LIT-47 (both from Merz-Dade). In addition, we analyzed our in-house human serum calibrator (A3), which had been stored frozen for about 10 months.

Reflotron calibrator set. A new set of liquid plasma calibrators (set 02, lot no. 02; 12 concentrations) was shipped from Mannheim to Rotterdam, packed in solid CO2, for measurement with the MAK Reference Method to obtain "certified reference method values." These samples are shock-frozen citrated human plasmas with added lipid fractions.

Protocol. On each of 10 working days 20 of our patients' sera were taken in such a way that a sufficient number of low and above-normal concentrations were included in our comparison. Because this selection took place in the afternoon, measurements were done the next day, the samples having been kept overnight at 4 °C. Samples of the lyophilized serum controls were included in the runs of 20 patients' samples. All samples were then analyzed once with the reference method, in duplicate with the standardized enzymatic method, and in duplicate with Reflotron test strips.

The three Reflotron analyzers were used sequentially, to provide insight into inter-instrument variability. Duplicates were measured in each instrument. In this way 66, 67, and 67 serum samples were analyzed in the respective three analyzers. The calibrator set of 12 samples was analyzed with the MAK Reference Method on 10 days, in duplicate. After thawing, there was some clotting in the samples with the highest cholesterol concentration.

Statistical analysis. We compared the MAK with the standardized enzymatic assay, and both of these with the Reflotron. We performed orthogonal regression analysis according to Cornbleet and Gochman (19) and regression analysis according to the model of Passing and Bablok (20). The results were practically identical. A convenient comparison was also obtained by looking at just the means of two series of results when the concentration range was too low to allow a meaningful regression equation.

Results

Analytical results for the 200 serum samples by the three different methods were subjected to orthogonal regression analysis. The version according to Passing and Bablok is shown (20): (y = ax + b)

**MAK = x**

\[ y = 0.980x + 0.003, \quad r = 0.994 \]

ChOD-PAP = y

\[ \text{Mean } x = 6.093 \]

\[ \text{Mean } y = 5.981 \text{ mmol/L} \]

**MAK = x**

\[ y = 0.985x + 0.238, \quad r = 0.989 \]

Reflotron = y

\[ \text{Mean } x = 6.093 \]

\[ \text{Mean } y = 6.251 \text{ mmol/L} \]

**ChOD-PAP = x**

\[ y = 1.009x + 0.203, \quad r = 0.989 \]

Reflotron = y

\[ \text{Mean } x = 5.981 \]

\[ \text{Mean } y = 6.251 \text{ mmol/L} \]

The individual equations for the three Reflotron analyzers are [all with MAK = x and Reflotron = y (y = ax + b):]

\[ \text{Mean } x \quad \text{Mean } y \quad \text{SD}_a \quad \text{SD}_b \]

\[ n = 67, \quad y = 0.993x + 0.187, \quad r = 0.991 \]

\[ 0.261, \quad r = 0.991 \]

\[ n = 66, \quad y = 0.970x + 0.019, \quad r = 0.999 \]

\[ 0.265, \quad r = 0.990 \]

\[ n = 65, \quad y = 0.985x + 0.203, \quad r = 0.989 \]

\[ 0.246, \quad r = 0.982 \]

The differences between the calculated means of the series show a slight positive bias for Reflotron, a combination of the slightly negative slope and the positive constant factor. The strip test yields cholesterol values about 2% to 4% too high in serum samples. The cutoff on the y-axis shows that low concentrations are overestimated slightly more than high ones. Figure 1 shows the three comparisons graphically.

Calibrator set 02, lot no. 02 was analyzed 10 times, in duplicate, with the MAK Reference Method. The overall analytical coefficients of variation (CV) for each of the 12 calibrator concentrations were 0.6% or 0.7% consistently. The mean findings are given in Table 2. The Passing–Bablok regression equation, with MAK = x and Reflotron = y, was \( y = 1.016x + 0.017 \) (\( r = 0.999 \)). The mean \( y = 5.323 \) and the mean \( x = 5.226 \text{ mmol/L} \). The standard deviations were: \( \text{SD}_a = 0.007 \) and \( \text{SD}_b = 0.05 \).

When the samples were thawed we observed some clotting in concentrations IX through XII. The calcium present in lipoprotein fractions added to the plasma facilitated this fibrin formation. When the entire fibrin clot from one of the vials of pool XI was analyzed with the MAK Liebermann–Burchard reagent, the amount of color indicated no significant presence, and thus no loss, of cholesterol.

The lyophilized serum controls. Reconstituted lyophilized, and also frozen serum as a rule, are not analyzed accurately in the Reflotron cholesterol test (21). Table 3 shows these differences in some materials. The CVs for the ChOD-PAP and Reflotron methods were 1% to 4% and 2% to 4%, respectively.
Discussion

When new analytical concepts are introduced, the question of analytical accuracy arises. In screening of large numbers of subjects, standardization becomes more essential than ever. Fortunately, for total cholesterol we have access to Definitive and Reference Methods with proven reliability (22). Because the Reflotron method (like other assays) is sensitive to the matrix of serum controls, one must use calibrators that mimic the average human serum matrix as closely as possible. When the calibration procedure yields accurate measurements for human serum or plasma, the result may be considered optimal. We have studied whether the manufacturers' calibration method is based on accurate target values and whether the calibration leads to accurate results in human serum.

The comparison in 200 fresh human serum specimens has shown a bias in the Reflotron method, but it has become very small. A small correlation in the calibration curve may improve the accuracy even further, particularly so in the lower concentration range. At present the performance of this cholesterol assay does not, in our hands, appear inferior to that of wet-chemical enzymatic procedures that are in common use in medical laboratories today (23). Comparability in finger-stick whole blood, possible interferences in the matrix, and lot-to-lot reagent reagentability are the subjects of present investigation.

We thank Prof. Dr. M. B. Katan (Wageningen Agricultural University), Dr. W. F. A. Groote (Rotterdam Academic Children's Hospital Sophia) and Dr. Reh (Boehringer Mannheim) for advice and stimulating discussions. Mr. G. J. Krünen (Boehringer Nederland BV, Almelo, The Netherlands) installed the three analyzers and provided logistical support. The Reference Laboratory of the Rotterdam University Hospital "Dijkzigt" operates in cooperation with the National Institute of Public Health & Environmental Hygiene, RIVM, Bilthoven (Dr. J. C. Koedam). We particularly acknowledge the cooperation of the Lipid Reference Center at CDC, Atlanta, GA (Drs. G. R. Cooper, A. Hainline Jr., G. L. Myers et al.). A part of this work was supported by the Netherlands Heart Foundation (Dr. W. Stiggelbout, director) through grant no. 86.003.

References
2. Marks V, Alberti KGMM. Clinical biochemistry nearer the

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<thead>
<tr>
<th>Table 2. Results of Analysis of Boehringer Mannheim (BM) Calibrator Set 02, Lot 02</th>
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<tbody>
<tr>
<td>Cholesterol concn, mmol/L</td>
</tr>
<tr>
<td>MAK</td>
</tr>
<tr>
<td>2.12*</td>
</tr>
<tr>
<td>BM</td>
</tr>
<tr>
<td>2.23</td>
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<tr>
<td>CV, %</td>
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<td>0.61</td>
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*Mean of 20 determinations.

<table>
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<tr>
<th>Table 3. Accuracy and Precision of the Cholesterol Assays with Commercial Serum Controls</th>
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</thead>
<tbody>
<tr>
<td>MAK</td>
</tr>
<tr>
<td>ChOD-PAP (n = 20)*</td>
</tr>
<tr>
<td>Reflotron (n = 20)</td>
</tr>
<tr>
<td>Pool*</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>PO2</td>
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<td>PNL</td>
</tr>
<tr>
<td>PNU</td>
</tr>
<tr>
<td>A3</td>
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<tr>
<td>LipTrol</td>
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</table>

*For pool identification see Methods and Materials.
*When n = 20, two values were obtained on each of 10 working days. When n = 6, single MAK values were obtained on different days.
Drugs Without GmbH, 4.


