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Standardization of Serum Cholesterol Assays by Use of Serum Calibrators and Direct Addition of Liebermann–Burchard Reagent

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Serum cholesterol concentrations of subjects in epidemiological studies were measured after direct addition of Liebermann–Burchard reagent; results were calibrated with human serum pools assayed according to Abell et al. (*J. Biol. Chem.* **195**: 357–366, 1952). Accuracy and precision were monitored for six years by analysis of internal-control pools and blind external-control pools. For various internal-control pools, the imprecision (CV) of the long-term averages of run means ranged from 0.5 to 0.9%. The within-run CV for internal control and patients' sera was about 1%. For blind control sera with different concentrations (provided by the Centers for Disease Control, Atlanta, GA, over the same period), the average difference per three-month period between the values found and the target values was usually between −0.5% and +0.7% for medium-concentration pools and between −2% and +2% for low- and high-concentration pools (extreme values: −2.4% and +2.5%). The CV per three-month period ranged from 0.6 to 2.7%. Sera from subjects on diets of high or low linoleic acid content were analyzed to study the effect of the fatty acid portion of serum cholesterol esters; the differences between values obtained with the comparison method and the direct method was insignificant on both diets. We conclude that the use of serum calibrators eliminates the bias inherent in the direct method.

Additional Keyphrases: *calibrators prepared from frozen serum* • *effect of dietary fatty acids on serum cholesterol values*

The Liebermann–Burchard reagent, in some modification or other (e.g., *1*), is widely used for determination of cholesterol in serum (*2*). However, its color yield per molecule differs for cholesterol in serum and for pure cholesterol in calibration solutions, presumably because most of the cholesterol in serum is present as esters of long-chain fatty acids and not as free cholesterol (*3*), or because the Liebermann–Burchard reagent interacts differently with the background matrix of serum and with the solvent of calibration solutions (*2*). With the reference method of Abell et al. (*4*), one circumvents these problems by extracting the cholesterol from serum and saponifying it, thereby bringing it into the same physical state as the material used for calibration; the color developed is therefore read against that of an identical background. However, this method is laborious and cannot easily be automated. The Lipid Research Clinics AutoAnalyzer methods involve extraction with isopropanol (*5*), and a serum calibrator is used to correct for remaining bias, e.g., from the presence of esterified cholesterol in the serum extract (*3*).

We have found that highly accurate and precise values for serum cholesterol can be obtained without extraction, by adding Liebermann–Burchard reagent directly to serum and using sera of known cholesterol concentration instead of cholesterol solutions for calibration. The cholesterol concentrations in the calibration sera were determined by the method of Abell et al. (*4*).

We have used the direct method successfully for determining serum cholesterol concentrations for several Dutch and international epidemiological and experimental studies (*6–10*).

Materials and Methods

Preparation of Pools and Collection of Sera

Serum from healthy human donors, obtained from blood banks of the Netherlands Red Cross, was filtered through a Seitz EKS adsorption filter in a Seitz 12188 2-L stainless-steel filter holder (Gezang & Co., 1014 BB Amsterdam, The Netherlands) under pressure of 2–3 atm. (roughly 200–300 kPa), treated with ethylmercurithiosalicylate (0.1 g/L of filtrate), and stored at −20 °C until further use. Until the end of 1976 we isolated a cholesterol-rich fraction from the serum according to Williams et al. (*11*); after that time we used ultracentrifugation, because in our hands it was simpler and yielded sera that were less turbid after storage. We prepared a cholesterol-rich concentrate as follows: from a 1-L bottle of frozen serum left at room temperature until about 0.5 L of it

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had thawed, we discarded the remaining ice and put the fluid, which had a cholesterol concentration of about 10 mmol/L (3.87 g/L) into several 38-mL centrifuge tubes. After centrifugation for 16 h with a Beckman Ti 60 angle rotor (Beckman Instruments, Inc., Fullerton, CA 92364) at 18 °C and 54 000 rpm (max., 300 000 × *g*) a yellow-orange low-density lipoprotein band was visible a few centimeters from the top of the tube. We collected about 5 mL of concentrate [cholesterol concentration about 25 mmol/L (9.67 g/L)] from each tube and used it immediately to prepare calibrators or control sera.

We prepared a low-cholesterol serum calibrator [concentration about 2.5 mmol/L (0.97 g/L)] by mixing human serum with horse serum (National Institute of Public Health RIV, 3721 MA Bilthoven, The Netherlands), because human serum of such low concentration is difficult to obtain. Calibrators of medium (about 6 mmol/L) and high (about 9 mmol/L) concentration were prepared by adding concentrate to human serum. The pools were filtered as described above, partitioned into 5-mL glass ampoules, and stored at −20 °C. Internal quality-control sera were prepared exactly like the medium- and high-concentration calibrators except that 2-mL ampoules were used. Normal control pools contained about 4 mmol of cholesterol per liter (1.74 g/L) and above-normal pools about 7.5 mmol/L (2.90 g/L). About 2.5 L of each serum calibrator and 1 L of control serum were prepared at a time. Throughout the period of use, sera generally were clear after thawing.

Blood samples from subjects were collected from an antecubital vein into an evacuated tube, and allowed to clot for about 1 h at room temperature. Serum was obtained by low-speed centrifugation, and stored at −20 °C. Occasionally we also stored samples at 4 °C for a few days, up to a week.

Determination of Cholesterol

We used the method of Abell et al. (*4*), with slight modifications, to determine the cholesterol content of calibrators. Two technicians analyzed four samples of each pool on five separate days. Each made up his or her own solutions. Typical CVs for the 40 samples were 1–2%, yielding standard errors of the mean of 0.16–0.32%. Abell values for subjects' samples were based on replicate determinations.

For routine cholesterol determinations, we used a Prestomatic Model 8 Automatic Chemical Analyser (Meyvis, 4614 VV Bergen op Zoom, The Netherlands), which involves vigorous mixing of 0.1 mL of sample (or air as a blank) with 5 mL of an ice-cold mixture of concd. H_2SO_4 (96% purity)/glacial acetic acid/acetic anhydride (1/3/6 by vol), stabilized with anhydrous Na_2SO_4, 20 g/L.

We placed samples into a tray that held 36 cups. Each tray held duplicate samples of low-, medium- and high-concentration calibrators, eight blanks, and single samples of patients' or control sera. Thus, results of the direct method represent single analyses. After incubation at 25 °C for 25 min a Wang 600 microcomputer recorded the absorbance of the calibrators, samples, or blanks in a 1-cm cuvette; we used a Vitatron UPS 200 photometer (Meyvis), equipped with a tungsten lamp and a 627 nm interference filter. A computer program corrected the absorbances for carryover, calculated a linear least-squares calibration line from the net absorbances of the six calibrators, and converted absorbances of samples into concentrations. Each tray thus yielded a separate calibration slope.

Quality-Control Procedures

A run consisted of the results of three to eight consecutive 36-cup trays; sometimes two runs were completed in a day, but one run per day was usual. Four samples each of a normal and an above-normal control pool were distributed throughout each run (*5*). When a new batch of control serum was introduced, the results of the first 20 runs were used for determining a provisional mean, standard deviation (SD), and mean daily range; more definite values were obtained after 50 runs. Analytical systems such as the one described here tend to show long-term "cycling effects," in which assayed values on control pools fluctuate above or below the mean for extended periods (*5*). To take this into account, we set new control limits after each additional 20 runs by recalculating the means and SDs for all runs performed up to that point. This practice was abandoned in 1980, when we realized that it was not in agreement with Lipid Research Clinics procedures (*5*).

Measurements on old and new control pools generally overlapped for at least 20 runs. Results were judged by criteria similar to those of the Lipid Research Clinics Program (*5*). Specifically, all results of a run were rejected and all samples were re-analyzed or declared lost if one or more of the following events occurred for either the normal or the above-normal control:

a. A single daily mean fell outside the 3 SD limits of the average of daily means.

b. Two successive daily means fell between the 2 SD and 3 SD limits.

c. Eight successive daily means fell either all above or all below the center line.

d. A single daily range (difference between highest and lowest of four) fell above the 3 SD limit for the average range.

e. Two successive range values fell between the upper 2 SD and 3 SD limits.

f. Eight successive range values fell above the center line.

Out-of-control data were recorded on the control charts but not used in any calculations.

Results

Internal Quality Control

Between January 1976 and June 1981 we used three batches of quality-control serum with a normal cholesterol concentration ranging from 4.08 to 4.72 mmol/L (1.58 to 1.83 g/L), and five above-normal pools with cholesterol ranging from 6.72 to 8.57 mmol/L (2.60 to 3.31 g/L). Analysis of control pools by the method of Abell et al. yielded values that averaged 1% higher (range: −0.3% to +3.7%) than the long-term averages obtained with the routine method.

Because pools differed in absolute concentration, SDs and ranges are expressed as percentages of concentration, so that different pools can be compared.

Over the period described, the CVs of the long-term averages of the run means of these eight internal-control pools ranged between 0.5 and 0.9%. As described above, during the first four years we recalculated averages of run means, SDs, and ranges every 20 runs, so as to avoid long series of rejected runs when the system was running for a long period above or below the value set after the first 50 runs. Quality-control limits were not systematically affected by this procedure. The maximum difference between a new average and the average set after the first 50 runs ranged from 0.0 to 0.3% for the above-normal control pools; these pools were usually exhausted in about a year. For two normal-concentration pools, which were used for two to three years each, the largest differences between a new target average and the average set after the first 50 runs were 0.7 and 0.9%.

The long-term average of the range, i.e., the difference between the highest and lowest value in one run, varied between 1.8 and 2.2% of the total concentration for the normal pools and between 1.6 and 1.9% for the above-normal pools, which

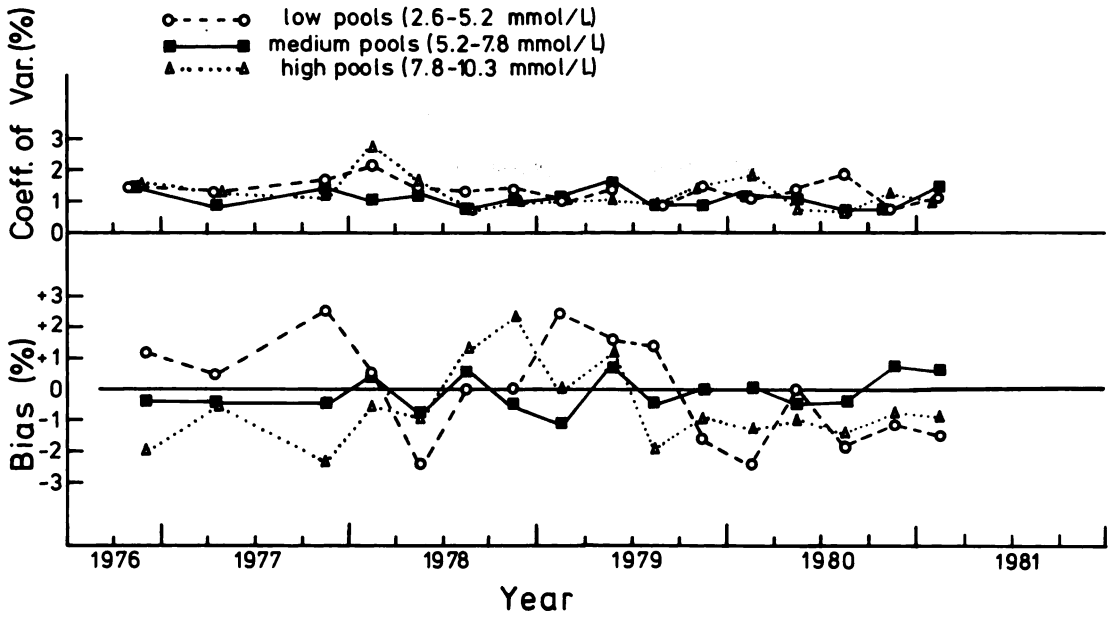

Fig. 1. Results for external-control sera in the Centers for Disease Control Cooperative Cholesterol Standardization Program

Upper curves: coefficients of variation (CVs). *Lower curves:* bias vs. CDC target values. For Phase II (November–December 1976) each point represents 20 samples. In Phase III (January–July 1977), five different pools, of 24 samples each, were analyzed; results of the two lowest and the two medium pools were combined for this graph. For Phase IV (starting October 1977), each point represents 18 samples

leads to estimates of a within-run CV (*12*) of 0.9 to 1.1% for the normal and 0.8 to 0.9% for the above-normal pools. Subtraction of this within-run component of variance from the variance of the long-term run means of control pools leaves a between-run CV of about 0.6% for the normal pools and about 0.5% for the above-normal pools.

The within-run CV was also calculated for patients' sera. Between 1977 and 1979, 332 of the samples collected in the Nijmegen Intervention Project were divided into two aliquots and submitted to us under blind codes. After the codes had been revealed we calculated a mean difference, d, between duplicates of 0.051 mmol/L (0.02 g/L) and a mode of 0.026 mmol/L (0.01 g/L). Application of the formula $SD = \sqrt{(\Sigma d^2/2n)}$ yielded a within-run SD of 0.054 mmol/L, corresponding to a CV of 1%, in excellent agreement with the values calculated above from the ranges of the control sera.

Altogether, 453 runs were completed in the period described; 44 of these had to be rejected. In addition, the laboratory was declared "out of control" for six weeks in 1978 because of a slight but persistent decrease in the mean of an above-normal control pool. After an overhaul of equipment and the introduction of a new control pool a stable pattern was again obtained.

External Quality Control

Figure 1 shows the accuracy and precision obtained for blind control sera provided by the Centers for Disease Control (CDC) under the CDC/WHO Cooperative Cholesterol and Triglyceride Standardization Program. Most points represent the mean of 18 samples of one pool, analyzed in three months. The upper part of Figure 1 shows the CV, a measure for precision. The lower part shows the difference between the mean found by us and the target value assigned by CDC; this is a measure for accuracy. Means and SDs were calculated by CDC from the individual values submitted by us. The inaccuracy (bias) oscillated around zero, rarely exceeding 1% for pools in the normal range of 5.2 to 7.8 mmol/L (2.00 to 3.00 g/L), or 2% for low or high-concentration pools. The CDC/WHO criteria for certification allow deviations of up to 5% from the target values. As Figure 1 shows, the CVs were 1–2%, again well within the CDC/WHO limits for imprecision: 0.07, 0.08, and 0.09 g/L for the low, medium, and high pools, respectively, corresponding to CVs of about 2.6 to 4.7%.

Interference Due to Cholesterol Ester Fatty Acid Composition

We wanted to test whether differences in the fatty acid portion of cholesterol esters caused differences in apparent cholesterol concentration according to the direct method. Therefore we applied both the direct method and the Abell method to sera of normolipemic subjects who had participated in a trial of diets either low or high in linoleic acid (*8*). These diets represented extremes of what can be achieved with commonly available foodstuffs and must have caused marked differences in serum cholesterol ester fatty acid composition within a few weeks (*13*, *14*). In 15 subjects who for five weeks ate a natural diet that provided 40% of energy as fat, 19% as *cis-cis* linoleic acid, and had a polyunsaturated:saturated fat (P:S) ratio of 1.7, the serum cholesterol concentration according to the direct method was 1.5 ± 0.9% (mean ± SEM) higher than the concentration determined by the Abell method. In 14 subjects who had consumed a diet that was identical except for its low linoleic acid content (3% of energy, P:S ratio 0.17), this difference was 2.8 ± 1.8%. Samples from both groups were analyzed together so as to minimize between-run effects. The differences did not differ significantly from each other or from zero.

Similar comparisons were also made for other subjects. Each subject was sampled once, and the serum was divided and analyzed by both the direct method and the Abell method. In a series of 141 sera of healthy school boys and girls (*6*, *7*), divided over 14 runs, the Abell values averaged 0.6% higher (*7*) than values obtained with the direct method. A series of 205 sera of adult men and women, analyzed in 10 different runs, showed on average 1.5% higher values by the Abell than by the direct method. The population from which these samples were obtained had a serum cholesterol (mean ± SD) of 5.3 ± 1.1 mmol/L (2.05 ± 0.42 g/L) for men and 5.0 ± 1.0 mmol/L (1.94 ± 0.38 g/L) for women.

Discussion

Our study shows that the inherent bias of the direct Lie-

bermann–Burchard method (*12, 15*) can be overcome by using serum calibrators and strict internal and external quality control. The idea of using calibrator sera to overcome this bias is not new (*16*). The Lipid Research Clinics AutoAnalyzer procedures also involve use of a serum calibrator to remove possible bias in the assay of isopropanol extracts of serum calibrated with isopropanol solutions of cholesterol (*3*). However, we are not aware of any previous long-term evaluation of the use of serum calibrators in a method where Liebermann–Burchard reagent is directly added to serum. Our results show that such a method can yield an accuracy and precision matching those obtained in the Lipid Research Clinics Program (*17, 18*), which probably represents the "state-of-the-art" in serum lipid assays.

The direct method is suitable for epidemiological studies of healthy subjects, but less so for clinical work, where jaundiced or lipemic samples may cause erroneous values (*2*). Differences in the ratio of free to esterified cholesterol in serum could also influence results, but this ratio is fairly constant in normal subjects. However, the proportion of the various fatty acids in the cholesterol esters can vary, and different cholesterol esters are reported to have different chromogenicity (*3*). We have tested this with sera from subjects who had consumed for five weeks diets either very high or low in linoleic acid (*8*). Such diets would cause large differences in the proportions of the various cholesterol esters in serum. If the chromogenicity of cholesterol linoleate is higher than that of cholesterol oleate, then one would expect erroneously high values for cholesterol in sera from high-linoleic acid subjects determined by the direct method. However, no such effect was observed, and we conclude that the difference in chromogenicity under our conditions was negligible.

Other analyses of unselected patients' sera by both the direct method and the method of Abell et al. also showed negligible differences. It is, of course, still possible that biased values were obtained in isolated cases, but in general the direct method appears to give Abell-equivalent values for healthy populations.

Although the method described in this paper is inherently simple and rapid, the overall procedure still had a low productivity—in part because of the time consumed in manufacturing and assaying pools, and, in part because of the large proportion of the analytical capacity given over to calibration and control samples. As a consequence, the cost per analysis was relatively high. However, such costs formed only a minor proportion of the total cost of the epidemiological research projects involved, and the value added to such projects by good standardization, in our opinion, far exceeded this expense.

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