Clinical Chemistry 44:4
779–789 (1998)

Lipids and Lipoproteins

Reference standardization and triglyceride interference of a new homogeneous HDL-cholesterol assay compared with a former chemical precipitation assay

Christa Cobbaert,[1*] Louwerens Zwang,[1] Ferruccio Ceriotti,[2] Annalisa Modenese,[2] Peter Cremer,[3] Wolfgang Herrmann,[4] Gerhard Hoss,[5] Jochen Jarausch,[5] Regina Türk,[5] Winfried März,[6] and Matthias Nauck[6]

A homogeneous HDL-c assay (HDL-H), which uses polyethylene glycol-modified enzymes and sulfated α-cyclodextrin, was assessed for precision, accuracy, and cholesterol and triglyceride interference. In addition, its analytical performance was compared with that of a phosphotungstic acid (PTA)/$MgCl_2$ precipitation method (HDL-P). Within-run CVs were ≤1.87%; total CVs were ≤3.08%. Accuracy was evaluated in fresh normotriglyceridemic sera using the Designated Comparison Method (HDL-H = 1.037 Designated Comparison Method + 4 mg/L; n = 63) and in moderately hypertriglyceridemic sera by using the Reference Method (HDL-H = 1.068 Reference Method − 17 mg/L; n = 41). Mean biases were 4.5% and 2.2%, respectively. In hypertriglyceridemic sera (n = 85), HDL-H concentrations were increasingly positively biased with increasing triglyceride concentrations. The method comparison between HDL-H and HDL-P yielded the following equation: HDL-H = 1.037 HDL-P + 15 mg/L; n = 478. We conclude that HDL-H amply meets the 1998 NCEP recommendations for total error; its precision is superior compared with that of HDL-P, and its average bias remains below ±5% as long as triglyceride concentrations are ≤10 g/L and in case of moderate hypercholesterolemia.

Several prospective epidemiologic studies and clinical trials have demonstrated that low concentrations of HDL-cholesterol (HDL-c)[7] are an independent risk factor for coronary heart disease, the inverse relationship being maintained over a wide range of HDL-c concentrations *(1, 2)*. It is estimated that for every 10 mg/L decrease in HDL-c, the relative risk for coronary disease events increases by 2–3% *(3)*. Also, the relationship appears to be equally strong in males and females, and among asymptomatic individuals as well as patients with established coronary disease. High HDL concentrations have been associated with longevity, whereas some, but not all, genetic deficiencies of HDL are associated with premature atherosclerosis *(4)*. Blood HDL-c concentrations are strongly influenced by family history and certain life-style factors, such as cigarette smoking, obesity, and physical inactivity. Frequently, low HDL concentrations are accompanied by high concentrations of triglycerides because of their metabolic interrelationship involving cholesterol ester transfer between HDL particles and triglyceride-rich lipoproteins *(5)*. Data from the PROCAM *(6)* and the Helsinki Heart *(7, 8)* studies further highlighted that the low HDL-c–hypertriglyceridemia syndrome is a powerful risk factor for nonfatal myocardial infarction or coronary artery disease death that would be overlooked if LDL-cholesterol concentrations alone were determined. Thus far, no completed clinical trial has been able to address the efficacy of raising HDL-c alone because life-style changes and lipid-active agents affect multiple lipids simultaneously.

[1] Academic Hospital Rotterdam, 3015 GD Rotterdam, The Netherlands.
[2] Istituto Scientifico H.S. Raffaele, Milan, Italy.
[3] Klinikum Grosshadern, Munich, Germany.
[4] Klinikum der Universität des Saarlandes, Hamburg, Germany.
[5] Boehringer Mannheim, Mannheim, Germany.
[6] Klinikum der Universität, Freiburg, Germany.
*Address correspondence to this author at: Department of Clinical Chemistry, Lipid Reference Laboratory, Academic Hospital Rotterdam, Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands. Fax 31-10 436 7894; e-mail: boersma@ckcl.azr.nl.
Received November 11, 1997; revision accepted January 9, 1998.

[7] Nonstandard abbreviations: HDL-c, HDL cholesterol; PEG, polyethylene glycol; PTA, phosphotungstic acid; CDC, Centers for Disease Control and Prevention; DCM, Designated Comparison Method; CFAS, Calibrator for Automated Systems; and NCEP, National Cholesterol Education Program.

Accordingly, HDL-c determinations are included in most national coronary heart disease prevention programs to predict an individual's risk and to guide treatment *(1, 9)*. As an example, the US National Cholesterol Education Program (NCEP) Adult Treatment Panel II has identified an HDL-c concentration <0.35 g/L as a major risk factor for coronary heart disease and considers HDL-c concentrations >0.60 g/L as a negative risk factor *(9)*. Because of the enhanced role of HDL-c in medical practice, reliable and easy-to-perform assays are warranted. Several techniques to determine blood HDL-c have been described, including ultracentrifugation, high-performance liquid chromatography, electrophoresis, and precipitation-based methods *(10)*. In routine clinical chemistry laboratories, HDL-c is frequently measured by means of chemical precipitation of the apolipoprotein B-containing lipoproteins with either polyethylene glycol (PEG), dextran sulfate, or PTA/$MgCl_2$, followed by quantitation of the cholesterol content in the HDL-containing supernate *(10)*. Standardization of these HDL-c assays is challenging because, in addition to matrix effects, substantial variability exists between the commonly used precipitation reagents *(11–13)*. In addition, in certain laboratories, precipitation methods do not meet the imprecision goal for usefulness of a medical test *(14)*. Finally, precipitation methods are time-consuming, require relatively large volumes of sample, and are not amenable to full automation.

Recently, a direct assay for HDL-c was developed in Japan *(15)* and commercialized by Boehringer. The assay is based on specific enzymatic hydrolysis of cholesterol esters and oxidation of cholesterol in HDL particles. The specificity is achieved by the use of sulfated α-cyclodextrin, PEG-modified cholesterol esterases and oxidases, and by optimization of pH and Mg^{2+} concentration *(15)*. Determinations can be made directly from serum or heparin plasma, without any pretreatment of samples. In this study, we aimed to evaluate this homogeneous HDL-c assay with respect to accuracy and traceability to the Reference Methods in two CDC Network Labs. A major focus was put on the accuracy evaluation in case of moderate and severe dyslipidemia in comparison with a conventional chemical precipitation method (PTA/$MgCl_2$). Furthermore, an extensive precision study was conducted in one CDC Network Lab. Finally, a field method comparison between the PTA/$MgCl_2$ precipitation method and the new homogeneous HDL-c method was performed in five university hospital laboratories.

Materials and Methods

PARTICIPATING LABORATORIES

Accuracy of the homogeneous HDL-c assay was investigated by two European members of the CDC Cholesterol Reference Method Laboratory Network, i.e., the Rotterdam group and the Milan group *(16)*. In Rotterdam, the CDC HDL-c Reference Method was used, whereas in Milan, the HDL-c Designated Comparison Method was performed. Precision and triglyceride interference were studied extensively in Rotterdam.

Five European university laboratories participated in the method comparison part of the study (Rotterdam, Milan, Munich, Freiburg, and Hamburg). In all centers, uniform protocols and identical lots of reagents, calibrators, and controls were used. Moreover, all centers were equipped with Hitachi 911 or Hitachi 717 analyzers (Boehringer Mannheim), and official Boehringer applications were used.

SPECIMENS

Because matrix limitations exist for HDL-c, the mechanism for transferring the accepted accuracy base involves comparisons using fresh human specimens. To this end, 4 × 6 mL venous whole blood was drawn from fasting blood donors (n = 41) and from dyslipidemic outpatients from the lipid clinic (n = 85), of whom informed consent was obtained to establish traceability of the homogeneous HDL-c assay to the HDL-c Reference Method, whereas 2 × 6 mL whole blood was sufficient for the reference standardization vs the Designated Comparison Method (n = 98 and 35, i.e., from blood donors and outpatients from the lipid clinic, respectively) *(10, 14)*. Blood was allowed to clot at room temperature, and serum was obtained by centrifugation at 1500*g* during 15 min. Subsequently, specimens were split and handled according to a standard CDC protocol; one aliquot was stored at 4 °C and analyzed with the direct HDL-c and PTA/$MgCl_2$-based method at the day of sample collection, and the other aliquot was stored at −70 °C for a maximum of 4 weeks until analysis with the Reference Methods took place.

In the cholesterol interference study (n = 35), the bias of both normocholesterolemic (n = 19) and hypercholesterolemic (n = 16) specimens was checked vs the HDL-c Designated Comparison Method. All specimens had serum triglycerides <2 g/L. In the triglyceride interference study (n = 85), sera with isolated hypertriglyceridemia (n = 32), with isolated hypercholesterolemia (n = 1), and with mixed hyperlipidemia (n = 52) were evaluated vs the HDL-c Reference Method.

For the method comparison between homogeneous and precipitation-based HDL-c assays, fresh native sera from 478 inpatients and outpatients, ~100 sera per center, that were neither icteric or hemolytic on visual inspection and had triglycerides <4 g/L, were collected from the hospital routine and analyzed. Direct and precipitation-based HDL-c determinations were performed in each participating center at the day of blood collection. HDL-c measurements were performed in singlicate in five independent assays.

HDL-C REFERENCE METHOD AND HDL-C DESIGNATED COMPARISON METHOD

Reference points for HDL-c measurements, as recommended by the NCEP Lipoprotein Measurement Working

group, are: (*a*) the CDC Reference Method, a three-step procedure involving ultracentrifugation, precipitation, and cholesterol analysis; and (*b*) the CDC Designated Comparison Method (DCM) *(10, 14, 16)*. The Reference Method combines removal of VLDL by a β-quantification ultracentrifugation procedure, isolation of HDL by precipitation of LDL from the β-quantification bottom fraction (d = 1.006 kg/L) using 46 mmol/L heparin-Mn^{2+}, and cholesterol analysis of the HDL-c supernate by the CDC-modified Abell-Kendall Reference Method *(17)*. The DCM involves isolation of HDL by dextran sulfate-Mg^{2+} precipitation, followed by cholesterol analysis with the Abell-Kendall method, and requires normotriglyceridemic sera because of its limited precipitation efficiency *(16)*.

In the multicenter part of the study, HDL-c measurements were performed in five analytical assays; the sera were analyzed in duplicate with the CDC Reference Method (n = 41) and the DCM (n = 98) and in singlicate with the homogeneous and precipitation-based HDL-c method. In the triglyceride interference study (n = 85), reference HDL-c analyses were done in duplicate and routine HDL-c analyses were done in singlicate.

homogeneous HDL-c assay

The reagents for the homogeneous HDL-c assay were obtained from Boehringer Mannheim. Reagent 1 was composed of 0.5 mmol/L α-cyclodextrin sulfate, 0.5 g/L dextran sulfate, 2 mmol/L $MgCl_2$, and 0.3 g/L *N*-ethyl-*N*-(3-methyl-phenyl)-*N′*-succinyl-ethylene diamine in 30 mmol/L 3-(*N*-morpholino)propane sulfonic acid buffer, pH 7.0. Reagent 2 contained PEG-coupled cholesterol esterase (≥1 kU/L), PEG-coupled cholesterol oxidase (≥5.6 kU/L), peroxidase (30 kU/L), and 4-aminophenazone (0.5 g/L) in 30 mmol/L 3-(*N*-morpholino)propane sulfonic acid buffer, pH 7.0. In the first step, 4 μL of sample was mixed with 300 μL of reagent 1 and incubated for 5 min at 37 °C. In a second step, 100 μL of reagent 2 was added and incubated for an additional 5 min. The resulting color was measured bichromatically at 600 nm (main wavelength) and 700 nm (subsidiary wavelength).

The first reagent (HDL homogeneous R1, lot 131AEJ, 75 mL) was ready for use. The second reagent was composed of a lyophilized component (HDL homogeneous R2a Lyo, lot 120 AEJ) to be reconstituted with an aliquot of R2 buffer (HDL homogeneous R2, lot 120 AEJ, 20 mL). After reconstitution, the reagents were kept on board of the Hitachi analyzers. According to the manufacturer, the stability of the reconstituted R2 reagent and calibration stability is up to 4 weeks at 4–8 °C. For this study, the reagent was calibrated at the time each assay was run. For calibration, a human-based calibrator (HDL/LDL calibrator high, lot ML01 0058) was used, which was preliminarily "targeted" by the manufacturer using the PTA/$MgCl_2$ precipitation method (assigned value, 0.704 g/L). The reconstituted calibration material is claimed to be stable up to 1 day at 4–8 °C.

To assess traceability to the Reference Methods, complete analytical systems should be evaluated (including instrument model, application, reagent lot, and calibrator lot). In this reference standardization study, the lot numbers displayed above were evaluated; the analyzers used were a Hitachi 911 in Rotterdam and a Hitachi 717 in Milan. For precision evaluation, two additional lots of reagent were investigated on a Hitachi 911 (kit lots were 135AEK and 67241101, respectively). For the bias survey in dyslipidemic specimens, calibrator lot 19079901 and reagent lot 68079401 were used.

HDL-c determination by a PTA/$MgCl_2$ precipitation method

The analytical performance of the homogeneous HDL-c method was compared with the performance of a conventional PTA/$MgCl_2$ precipitation method from the same manufacturer (Boehringer Mannheim, cat. no. 543003, lot 660393-01). The PTA/$MgCl_2$-based assay was calibrated with 3.5-fold diluted Calibrator for Automated Systems (CFAS, Boehringer Mannheim, cat. no. 759350, lot 184927) in all clinical laboratories, according to the manufacturer's instruction (denoted as PTA/$MgCl_2$[CFAS]). To precipitate apolipoprotein B-containing lipoproteins, 200 μL of serum was mixed with 500 μL of precipitating reagent, composed of 0.44 mmol/L PTA and 20 mmol/L $MgCl_2$. The mixture was allowed to stand 5 min and was subsequently centrifuged (3000*g*, 10 minutes, 20 °C). Cholesterol in HDL was determined with the Boehringer Mannheim cholesteroloxidase-phenol-aminophenazone (CHOD-PAP) reagent (cat. no. 1489437, lot 664358-01).

HDL-c determination by a combined ultracentrifugation and PTA/$MgCl_2$ precipitation method

Laboratories 3 and 4 performed a combined ultracentrifugation and precipitation assay as an additional comparison method. Hitherto, the protocol of the Lipid Research Clinics Program was followed, with modifications published previously *(18)*.

triglyceride assay

Triglycerides were determined with a glycerolphosphateoxidase-phenol-aminophenazone method (GPO-PAP) (Boehringer Mannheim, cat. no. 1058550) on Hitachi 911 or Hitachi 717 analyzers. The application with Selective Mode Solution (i.e., 200 mmol/L HCl; Boehringer Mannheim, cat. no. 1360922) as R2 was used, according to the manufacturer's protocol.

analyzers

Homogeneous and precipitation-based HDL-c determinations were performed on Hitachi 911 (n = 4) or Hitachi 717 (n = 1) analyzers (Boehringer Mannheim) in each participating center. Daily maintenance and operation was performed according to the instructions of the manufacturer.

CONTROL SERA

Precision studies were performed by using processed quality control materials from Boehringer Mannheim: Precinorm^R L (lots 185597 and 186586) and Precipath^R L neu (lots ML01 0062 and ML02 2716). Besides, several batches of fresh frozen human sera at three concentrations (low, medium, and high) were examined.

DATA ANALYSIS

Before starting the multicenter data analysis, it was verified whether control sera were within the preset control limits (mean ± 3 SD). In the multicenter part of the study, 1 of 25 homogeneous HDL-c assays had to be omitted (lab 2, first run, n = 19).

In case of up to 200 paired data points, regression analyses were performed according to the method of Passing and Bablok *(19)*; in the case that more than 200 data points had to be compared, orthogonal regression was used. Total error (%) of the HDL-c assays was calculated as follows: [(1.96 × analytical imprecision (%)) + absolute mean bias (%)] *(14)*. Calculation of mean bias and total error vs the DCM was, according to CDC recommendations, based solely on specimens with triglycerides ≤2 g/L *(16)*. In case of the PTA/$MgCl_2$ precipitation assay, only data from clear and undiluted supernates, reflecting complete precipitation of apolipoprotein B-containing lipoproteins, were included in the final data analysis.

Results

PRECISION

Within-run imprecision data (n = 21) in processed control materials are presented in Table 1. Precision evaluation of the homogeneous HDL-c assay according to NCCLS EP5-T guidelines *(20)* was performed in the CDC Network Laboratory of Rotterdam (Table 2). Hitherto, duplicate HDL-c determinations per specimen per assay and two assays per day for 21 days were analyzed. Between-run imprecision data, gathered by the participating centers during the multicenter part of the study and based on duplicate determinations in lyophilized control materials and frozen human serum pools, respectively, demonstrate good precision of the evaluated HDL-c methods at that time (Tables 3 and 4).

BIAS VS THE CDC ULTRACENTRIFUGATION REFERENCE METHOD

The regression equations of homogeneous precipitation-based HDL-c method vs the CDC Reference Method are

Table 1. Within-run imprecision data (n = 21) of the Boehringer homogeneous HDL-c method compared with the Boehringer PTA/$MgCl_2$ precipitation method.

		Homogeneous HDL-c method[a]		PTA-$MgCl_2$ method[a]	
Laboratory	Analyzer	PNL lot 186 586	PPL neu lot ML02 2716	PNL lot 186 586	PPL neu lot ML02 2716
Lab 1	Hitachi 911	429 ± 5 (1.23%)	289 ± 3 (1.18%)	409 ± 4 (1.03%)	265 ± 4 (1.33%)
Lab 2	Hitachi 717	436 ± 5 (1.18%)	293 ± 3 (1.12%)	429 ± 7 (1.55%)	260 ± 4 (1.43%)
Lab 3	Hitachi 911	428 ± 4 (0.84%)	286 ± 1 (0.21%)	437 ± 6 (1.36%)	285 ± 3 (1.17%)
Lab 4	Hitachi 911	469 ± 3 (0.54%)	325 ± 2 (0.72%)	412 ± 7 (1.71%)	275 ± 6 (2.23%)
Lab 5	Hitachi 911	441 ± 3 (0.60%)	301 ± 2 (0.82%)	402 ± 3 (0.69%)	268 ± 1 (0.44%)

The data were produced in a Dutch, an Italian, and three German clinical laboratories (n = 5) using lyophilized quality control materials.
[a] Mean ± SD (mg/L) (CV, %)

Table 2. Precision evaluation of the homogeneous HDL-c assay according to NCCLS EP5-T.

	Within-run CV, %[a] (n = 21 replicates)			Total CV, %[a] (n = 21 runs)		
HDL-c lot number	Low	Medium	High	Low	Medium	High
R1: 131AEJ	28.86 ± 0.54	42.86 ± 0.43		28.89 ± 0.63	42.64 ± 0.80	
R2: 120AEJ	(1.87%)	(1.00%)		(2.18%)	(1.88%)	
R1/R2: 135AEK	29.15 ± 0.34	47.92 ± 0.32	85.61 ± 0.64	28.53 ± 0.69	46.65 ± 1.00	83.07 ± 1.68
	(1.17%)	(0.67%)	(0.75%)	(2.42%)	(2.14%)	(2.02%)
R1/R2: 67241101	30.42 ± 0.21	46.14 ± 0.33	69.15 ± 0.39	31.12 ± 0.96	47.08 ± 1.31	70.18 ± 1.61
	(0.69%)	(0.72%)	(0.56%)	(3.08%)	(2.78%)	(2.29%)

Three reagent lots were examined. Either lyophilized controls (in combination with lot number 131AEJ for R1 and lot number 120AEJ for R2) or fresh frozen pools (for kit lot numbers 135AEK, respectively; reagent lot 67241101) at different HDL-c levels were evaluated.
[a] Mean ± SD (mg/L) (CV, %).

Table 3. Overall imprecision data (five runs; duplicate determinations per run) for the Boehringer homogeneous HDL-c method compared with the Boehringer PTA/$MgCl_2$ precipitation method.

		Homogeneous HDL-c method[a]			
Laboratory	Analyzer	PNL lot 185 597	PNL lot 186 586	PPL neu lot ML02 0062	PPL neu lot ML02 2716
Lab 1	Hitachi 911	488 ± 5 (0.95%)	435 ± 4 (0.84%)	331 ± 7 (2.26%)	300 ± 6 (2.10%)
Lab 2	Hitachi 717	486 ± 7 (1.53%)	444 ± 8 (1.72%)	332 ± 6 (1.93%)	304 ± 4 (1.32%)
Lab 3	Hitachi 911	477 ± 6 (1.34%)	431 ± 3 (0.70%)	321 ± 5 (1.58%)	292 ± 6 (1.96%)
Lab 4	Hitachi 911	512 ± 7 (1.40%)	463 ± 4 (0.93%)	355 ± 5 (1.30%)	322 ± 7 (2.21%)
Lab 5	Hitachi 911	488 ± 10 (2.06%)	449 ± 11 (2.42%)	338 ± 8 (2.42%)	307 ± 4 (1.34%)
		PTA-$MgCl_2$ method[a]			
Lab 1	Hitachi 911	457 ± 7 (1.52%)	418 ± 6 (1.42%)	301 ± 5 (1.72%)	268 ± 4 (1.36%)
Lab 2	Hitachi 717	442 ± 6 (1.28%)	408 ± 8 (2.08%)	291 ± 5 (1.55%)	260 ± 15 (5.82%)
Lab 3	Hitachi 911	482 ± 28 (5.87%)	436 ± 17 (3.83%)	318 ± 21 (6.59%)	286 ± 16 (5.49%)
Lab 4	Hitachi 911	467 ± 13 (2.72%)	430 ± 8 (1.78%)	313 ± 8 (2.52%)	288 ± 3 (1.20%)
Lab 5	Hitachi 911	441 ± 6 (1.34%)	417 ± 8 (2.03%)	292 ± 9 (3.05%)	264 ± 9 (3.34%)

The data were produced in a Dutch, an Italian, and three German clinical laboratories (n = 5) using lyophilized quality control materials.
[a] Mean ± SD (mg/L) (CV, %).

presented in Table 5 (HDL-c range, 0.07–0.799 g/L; triglyceride range, 0.44–3.94 g/L). For the homogeneous HDL-c method (*Y*) vs the CDC Reference Method (*X*) the slope of the regression equation is significantly higher than one at $\alpha = 0.05$, whereas the intercept is not significantly different from zero. Fig. 1, A and B, demonstrates a mean bias of 2.2% for the homogeneous HDL-c method and −3.9% for the PTA/$MgCl_2$ assay, respectively. Assuming that the overall imprecision of the homogeneous HDL-c assay is maximally 3.08% (Table 2), the total error of the assay is 8.2%.

bias vs the designated comparison method

The regression equations of homogeneous precipitation-based HDL-c assays vs the DCM are presented in Table 5 (HDL-c range, 0.186–1.052 g/L; triglycerides, ≤2 g/L; n = 63). Analogously, the slope of the regression equation for the homogeneous HDL-c method (*Y*) vs the CDC DCM (*X*) is significantly higher than one at $\alpha = 0.05$. Fig. 1, C and D, demonstrates a mean bias of 4.5% for the homogeneous HDL-c method and −5.0% for the PTA/$MgCl_2$ assay, respectively. Assuming an overall imprecision of maximally 3.08% (Table 2) for the homogeneous assay, the total error of the method is 10.5%.

method comparison with the PTA/$MgCl_2$ precipitation method

The results of the method comparison between homogeneous and precipitation-based HDL-c methods are presented in Table 6 (HDL-c range, 0.07–1.25 g/L; triglycerides, ≤4 g/L). From the individual and pooled regression equations, it can be seen that the homogeneous HDL-c assay produced results that were significantly higher ($P < 0.05$) than those produced with the PTA/$MgCl_2$ assay. The same holds for laboratories 3 and 4, which used the combined ultracentrifugation and precipitation method.

cholesterol interference

In Fig. 2, A and C, it is illustrated that the homogeneous HDL-c assay does not become biased in the case of hypercholesterolemia. All specimens examined here (n = 35) were normotriglyceridemic (triglycerides <2 g/L), whereas 16 specimens were hypercholesterolemic (cholesterol >2.4 g/L). The cholesterol range tested varied between 1.63 and 3.91 g/L, whereas average and median biases were 2.5% and 3.0%, respectively (range, −3.0% to 10.5%). In each of the 35 samples examined, the bias of the homogeneous HDL-c method was less than ±13%.

In Fig. 2, B and D, the biases of the PTA/$MgCl_2$

Table 4. Interlaboratory survey (five runs; duplicate determinations per run) of the Boehringer homogeneous HDL-c method and the Boehringer PTA/$MgCl_2$ precipitation method in five clinical laboratories using frozen human serum samples.

		Homogeneous HDL-c method[a]		
Laboratory	**Analyzer**	**HS1**	**HS2**	**HDL-c 114AEJ 4.96**
Lab 1	Hitachi 911	644 ± 15 (2.40%)	364 ± 10 (2.63%)	504 ± 6 (1.10%)
Lab 2	Hitachi 717	663 ± 11 (1.72%)	381 ± 10 (2.63%)	519 ± 5 (1.00%)
Lab 3	Hitachi 911	648 ± 13 (1.98%)	363 ± 10 (2.66%)	508 ± 10 (1.91%)
Lab 4	Hitachi 911	703 ± 11 (1.60%)	413 ± 7 (1.59%)	561 ± 8 (1.47%)
Lab 5	Hitachi 911	660 ± 12 (1.76%)	373 ± 9 (2.44%)	514 ± 8 (1.60%)
		PTA-$MgCl_2$ method[a]		
Lab 1	Hitachi 911	626 ± 4 (0.58%)	348 ± 3 (0.80%)	473 ± 6 (1.32%)
Lab 2	Hitachi 717	622 ± 6 (1.04%)	351 ± 21 (6.06%)	470 ± 8 (1.77%)
Lab 3	Hitachi 911	666 ± 23 (3.43%)	376 ± 16 (4.13%)	489 ± 28 (5.74%)
Lab 4	Hitachi 911	695 ± 32 (4.55%)	408 ± 17 (4.18%)	502 ± 11 (2.27%)
Lab 5	Hitachi 911	606 ± 12 (2.03%)	324 ± 8 (2.50%)	459 ± 7 (1.48%)

[a] Mean ± SD (mg/L) (CV, %).

precipitation method are displayed as a function of HDL-c and cholesterol concentration, respectively. Average and median biases were −1.9% and −2.0%, respectively (range, −9.4% to 4.5%). Analogously, no bias could be demonstrated across the cholesterol range tested.

TRIGLYCERIDE INTERFERENCE

In Fig. 3, A and C, it is illustrated that the homogeneous HDL-c assay becomes positively biased in case of severe hypertriglyceridemia, a condition frequently encountered in conjunction with low HDL-c concentrations. The triglyceride range tested varied between 2 and 59 g/L. In case of triglyceride concentrations ≤20 g/L (n = 78), average and median biases were 4.3% and 3.7%, respectively (range, −26.3% to 47.2%). In all specimens with triglycerides >20 g/L, huge positive biases were demonstrated for the homogeneous HDL-c method, ranging between 51% and 578%. At triglyceride concentrations ≤10 g/L, the bias of the homogeneous HDL-c method was less than ±13% in 60 of 66 samples; from 10 g/L on, unacceptable triglyceride interference could eventually appear.

In Fig. 3, B and D, the biases of the PTA/$MgCl_2$ precipitation method are displayed as a function of HDL-c and triglyceride concentration, respectively. Overall, a tendency to an increasingly negative bias with increasing triglyceride concentrations existed, especially >10 g/L of triglycerides. If triglycerides were ≤20 g/L (n = 78), average and median biases were −7.3% and

Table 5. Reference standardization of field HDL-c methods.

CRMLN[a] laboratory	Y method	X method	n	Slope (95% CI)	Intercept (95% CI), mg/L	Correlation coefficient	Mean X, mg/L	Mean Y, mg/L
Rotterdam	Homogeneous HDL-c	Reference Method	41	1.068[b] (1.018–1.105)	−17 (−33–+5)	0.993	501	514
Rotterdam	PTA/$MgCl_2$	Reference Method	41	1.053[b] (1.012–1.092)	−41[b] (−61–−21)	0.988	501	487
Milan	Homogeneous HDL-c	DCM[c]	79	1.020[b] (1.000–1.042)	14[b] (+5–+24)	0.996	489	511
Milan	Homogeneous HDL-c	DCM[d]	63	1.037[b] (1.010–1.065)	4 (−10–+18)	0.996	519	541
Milan	PTA/$MgCl_2$	DCM[c]	98	1.002 (0.989–1.017)	−17[b] (−26–−13)	0.996	509	489

Regression analyses were done according to Passing and Bablok *(19)*.

[a] CRMLN, Cholesterol Reference Method Laboratory Network; CI, confidence interval.

[b] Slope and/or intercept are significantly different from, respectively, one and zero at $\alpha = 0.05$.

[c] Includes sera with triglycerides between 2 and 4 g/L.

[d] Includes only sera with triglycerides ≤2 g/L.

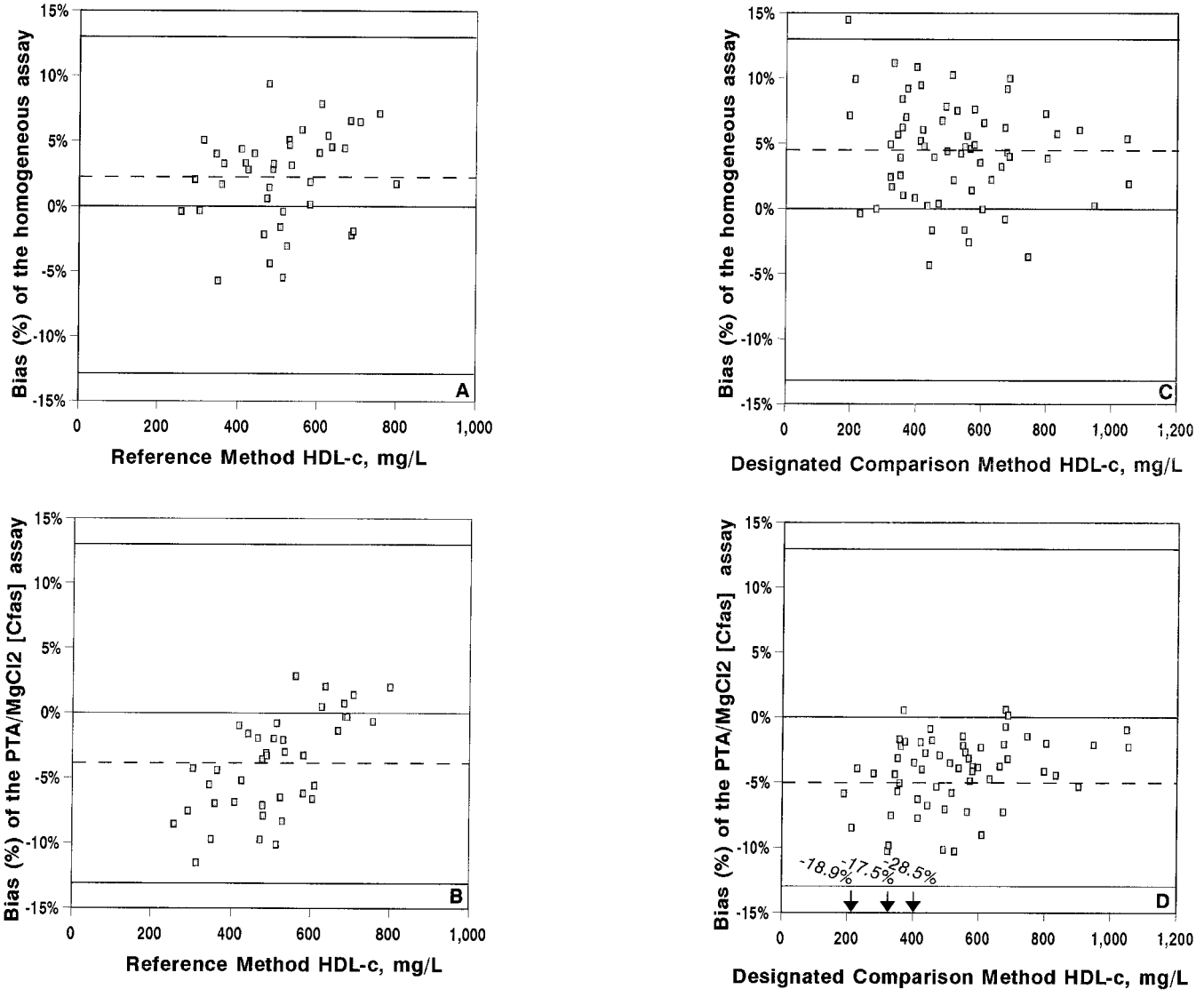

Fig. 1. Reference standardization study.

Percentage of bias of fresh, native, normo- and moderately hypertriglyceridemic sera as measured with the homogeneous HDL-c assay (*A* and *C*) and the PTA/$MgCl_2$ precipitation assay (*B* and *D*) vs the HDL-c Reference Methods. The dotted line represents the mean percentage of bias of the field HDL-c methods (*A*, 2.2%; *B*, −3.9%; *C*, 4.5%; *D*, −5.0%); the *full lines* represent the maximum bias related to the 1998 NCEP total error goal of ±13% for singlicate HDL-c measurements.

−7.5%, respectively (range, −31.1% to 8.5%). In the case of triglycerides >30 g/L (n = 4), it was no longer possible to get a clear and homogeneous supernatant after precipitation of apolipoprotein B-containing lipoproteins, not even after making a twofold sample predilution with physiological saline solution. Of the 81 remaining samples in which a chemical precipitation was performed, five samples demanded predilution to get a clear supernatant.

Discussion

Working with manufacturers is an effective means, with great impact, for standardizing clinical analytes within the clinical laboratory community. To this end, the Cholesterol Reference Method Laboratory Network of the CDC implemented in 1994 a program for manufacturers to evaluate the accuracy of (HDL)-cholesterol methods *(14, 16)*. The program is based on the analysis of fresh patient samples with both field and Reference Methods, because the traditional approach of using processed materials for evaluation of accuracy is inherently flawed due to matrix effects that cause processed materials to assay differently from patient samples on some instrument systems. Aims of this study were to assess the accuracy of the newly developed homogeneous HDL-c method from Boehringer in both normo- and dyslipidemic sera and to compare its analytical performance and its robustness to that of a PTA/$MgCl_2$ precipitation method.

In 1995, the NCEP Working Group on Lipoprotein Measurement issued performance guidelines for HDL-c measurements that clinical laboratories should achieve by 1998 *(14)*. In the case of HDL-c, the percentage of total error of routine HDL-c determinations should be reduced

Table 6. Summary of field method comparisons.

Lab	Y method	X method	n	Slope (95% CI)[a]	Intercept (95% CI), mg/L	Correlation coefficient	Mean X, mg/L	Mean Y, mg/L
	Homogeneous HDL-c	PTA/$MgCl_2$ [CFAS][b]						
1			99	1.021 (0.998–1.047)	12[c] (3–21)	0.994	429	451
2			79	1.016 (0.991–1.047)	36[c] (22–49)	0.992	466	510
3			100	1.024 (0.990–1.060)	4 (−13–22)	0.988	511	528
4			100	1.095 (1.069–1.124)	−8 (−19–7)	0.992	422	453
5			100	1.018 (0.980–1.057)	39[c] (20–56)	0.985	508	557
Overall			478	1.037[c] (1.022–1.052)	15[c] (7–22)	0.987	468	499
	Homogeneous HDL-c	UC + PTA/$MgCl_2$ [CFAS]						
3 and 4			200	1.059[c] (1.034–1.082)	12[c] (3–21)	0.986	451	490

Regression analyses were done according to Passing and Bablok (19) if n ≤ 200 and using orthogonal regression if n > 200.

[a] CI, confidence interval.

[b] PTA/$MgCl_2$ [CFAS]: PTA/$MgCl_2$ assay standardized with CFAS.

[c] Slope and/or intercept are significantly different from, respectively, one and zero at $\alpha = 0.05$.

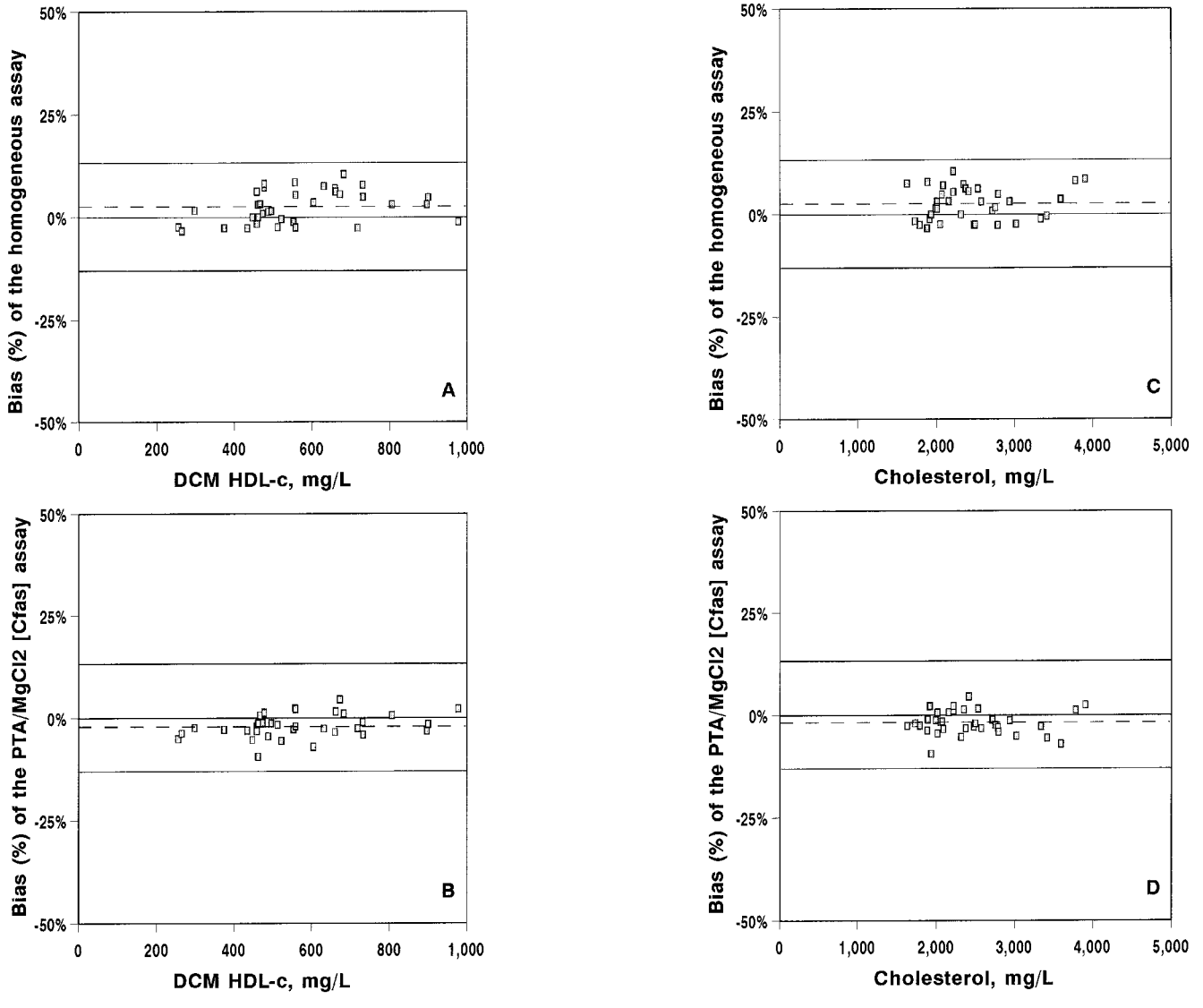

Fig. 2. Cholesterol interference study.

Percentage of bias of fresh, native, normo- and hypercholesterolemic sera as measured with the homogeneous HDL-c assay (*A* and *C*) and the PTA/$MgCl_2$ precipitation assay (*B* and *D*) vs the HDL-c Designated Comparison Method in relation to HDL-c and cholesterol concentrations, respectively. All sera were normotriglyceridemic (triglycerides <2 g/L). The *dotted line* represents the mean percentage of bias of the field HDL-c methods for all specimens evaluated (n = 35); the *full lines* represent the maximum bias related to the 1998 NCEP total error goal of ±13% for singlicate HDL-c measurements.

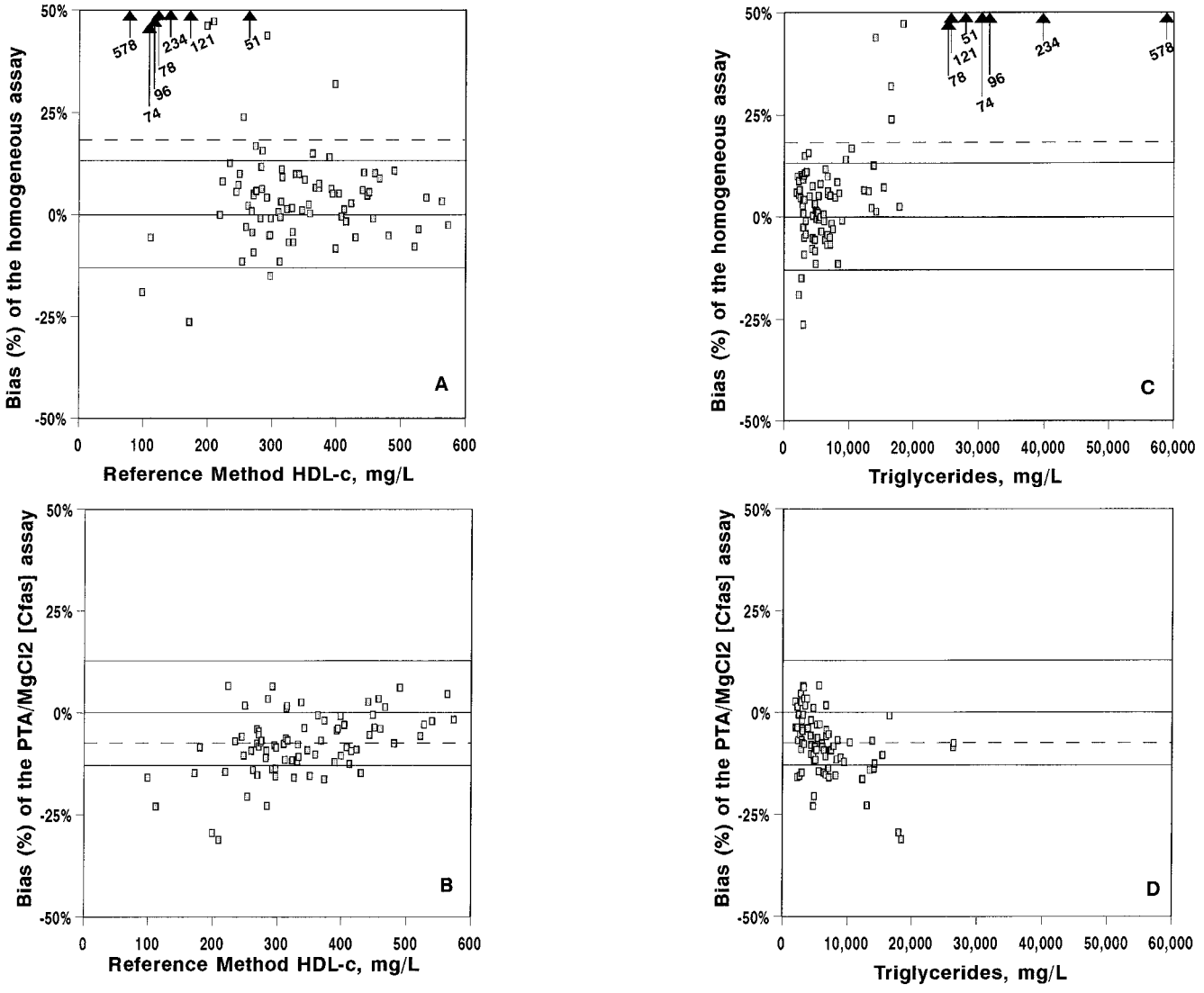

Fig. 3. Triglyceride interference study.

Percentage of bias of fresh, native, hypertriglyceridemic sera as measured with the homogeneous HDL-c assay (*A* and *C*) and the PTA/$MgCl_2$ precipitation assay (*B* and *D*) vs the HDL-c Reference Method in relation to HDL-c and triglyceride concentrations, respectively. The *dotted line* represents the mean percentage of bias of the field HDL-c methods for all specimens evaluated (homogeneous method, n = 85; PTA/$MgCl_2$ method, n = 81); the *full lines* represent the maximum bias related to the 1998 NCEP total error goal of ±13% for singlicate HDL-c measurements.

to ≤13%. Per definition, total error can be interpreted as an error budget one can divide between analytical imprecision (CV_a) and average bias. One set of conditions that is consistent with the total error goal is that at an HDL-c concentration of ≥0.42 g/L the CV_a should be ≤4% and the average bias less than or equal to ±5%.

In this study, it is illustrated that the homogeneous HDL-c assay, applied on Hitachi type analyzers, has an excellent reproducibility; total CVs ranged between 1.88% and 3.08% and were similar among different reagent lots (Table 2). From the field method comparison study, it became obvious that total CVs of the PTA/$MgCl_2$ assay differed substantially among laboratories in both lyophilized and frozen control materials (Tables 3 and 4). For example, CVs ranged between 1.36% and 6.59% in processed controls and between 0.58% and 6.60% in frozen sera, signifying that not all laboratories that use the PTA/$MgCl_2$ assay are able to reach the 1998 imprecision goal *(14)*. In contrast, total CVs of the homogeneous HDL-c method were less by one-half and much more consistent among participating centers, ranging between 0.70% and 2.26% in lyophilized controls and between 1.00% and 2.66% in fresh frozen sera. From the precision data, it is evident that the precision of the homogeneous HDL-c assay amply meets the 1998 NCEP requirements. In addition, the random error of the homogeneous HDL-c assay fulfills the generally accepted criterion for usefulness of a medical test, stating that the CV_a should be no greater than one-half the average biological variation (the biological CV of HDL-c is usually considered to be 7.5%) *(14)*.

The bias survey in normo- and moderately hypertriglyceridemic sera displayed a positive mean bias of similar magnitude in either CDC Network Laboratory (Table 5; Fig. 1, A and C). Notwithstanding the presence of a significant positive bias, the homogeneous assay met the 1998 total error goal, suggesting acceptable calibration of the method and adequate specificity for HDL, at least in normo- and moderately hypertriglyceridemic specimens. Analogously, results of the field method comparison (Table 6) demonstrated a positive bias of the homogeneous HDL-c assay compared with the PTA/$MgCl_2$ assay. The systematic difference is explained, first, by the arbitrary initial value setting of the homogeneous HDL-c calibrator, and second, because the CFAS calibrator of the PTA/$MgCl_2$ assay is value assigned by the Definitive Method, which produces results that are approximately 1.5% lower compared with those produced with the Reference Method *(16)*.

As CDC Network Laboratories strive to assist manufacturers in documenting and improving their method accuracy *(14, 16)*, a calibrator reassignment was recommended for the homogeneous assay. Accordingly, Boehringer Mannheim adjusted the calibrator value to 97% of its preliminary target value. Assuming a maximum CV_a of 3.08% (Table 2), the calibrator reassignment implies that total error of future homogeneous HDL-c measurements will be about 5.2% to 7.5% instead of 8.2% to 10.5%.

By means of the reference standardization part of the study, traceability of the Boehringer homogeneous HDL-c assay to the CDC Reference Methods has been established. Of course, the results presented here only apply to the specific analytical system evaluated (instrument model, reagent lot, and calibrator lot) and do not guarantee accuracy of future reagent and calibrator lots. Notwithstanding, conventional in-house quality control procedures at the manufacturer's site should be adequate to monitor the system in the future. If shifts are observed or suspected, another direct comparison with the HDL-c Reference Methods should be undertaken to reset the system for optimal accuracy.

The comparative bias surveys of conventional and direct HDL-c assays using fresh, dyslipidemic sera illustrate that the new Boehringer homogeneous HDL-c assay is as robust as the PTA/$MgCl_2$ precipitation method up to at least 3.91 g/L serum cholesterol (Fig. 2) and up to 10 g/L of serum triglycerides (Fig. 3). Above 10 g/L of serum triglycerides, the homogeneous HDL-c assay suffers from serious nonspecificity, leading to overestimation of the HDL-c concentration, whereas the PTA/$MgCl_2$ precipitation method becomes increasingly negatively biased. The observed percentage of bias largely varied among individuals having similar triglyceride concentrations and likely illustrates an effect of lipoprotein composition (Fig. 3, C and D). Whether sample predilution or the use of a reduced sample volume (3 μL in stead of 4 μL) could reduce bias in grossly dyslipidemic sera by using the Boehringer homogeneous HDL-c assay warrants further investigation.

We conclude that the new homogeneous HDL-c assay amply meets the 1998 NCEP recommendations for total error; its precision is superior compared with the PTA/$MgCl_2$ precipitation method, and its average bias remains well below 5% in case of moderate, isolated hypercholesterolemia or as long as serum triglyceride concentrations are $\leq$10 g/L.

We thank all technical assistants of the participating laboratories who were involved in this study for doing the analytical work. We also thank Boehringer Mannheim, Germany, for providing test kits free of charge.

References

1. Lewis B, Assmann G, Tikkanen M, Mancini M, Pometta D. Prevention of coronary heart disease: scientific background and new clinical guidelines. Recommendations of the European Atherosclerosis Society, prepared by the International Task Force for Prevention of Coronary Heart Disease. Nutr Metab Cardiovasc Dis 1992;2:113–56.
2. NIH. Consensus Development Panel on Triglyceride, High Density Lipoprotein, and Coronary Heart Disease. JAMA 1993;269:505–10.
3. Gordon DJ, Probstfield JL, Garrison RJ, Neaton JD, Castelli WP, Knoke JD, et al. High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. Circulation 1989;79:8–15.
4. Barter PJ, Rye KA. High-density lipoproteins and coronary heart disease. Atherosclerosis 1996;121:1–12.
5. Taskinen MR. Insulin resistance and lipoprotein metabolism [Review]. Curr Opin Lipidol 1995;6:153–60.
6. Assmann G, Schulte H. Relation of high-density lipoprotein cholesterol and triglycerides to incidence of atherosclerotic coronary artery disease (the PROCAM experience). Am J Cardiol 1992;70:733–7.
7. Huttunen JK, Manninen V, Mänttäri M, Koskinen P, Romo M, Tenkanen L, et al. The Helsinki Heart Study: central findings and clinical implications. Ann Med 1991;23:155–9.
8. Manninen V, Tenkanen L, Koskinen P, Huttunen JK, Manttari M, Heinonen OP, Frick MH. Joint effects of serum triglycerides and LDL cholesterol and HDL cholesterol concentrations on coronary heart disease risk in the Helsinki Heart Study. Am J Cardiol 1992;70:733–7.
9. Summary of the second report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Adult Treatment Panel II. JAMA 1993;269:3015–23.
10. Wiebe DA, Warnick GR. Measurement of High-density lipoprotein cholesterol concentration. In: Rifai N, Warnick GR, eds. Laboratory measurement of lipids, lipoproteins and apolipoproteins. Washington, DC: AACC Press, 1994:91–105.
11. Demacker PNM, Vos-Janssen HE, Hijmans AGM, van't Laar A, Janssen AP. Measurement of HDL-cholesterol in serum: comparison of six isolation methods with enzymatic cholesterol analysis. Clin Chem 1980;26:1780–6.
12. Warnick GR, Cheung MC, Albers JJ. Comparison of current methods for high-density lipoprotein cholesterol quantification. Clin Chem 1979;25:596–604.
13. Crook D. A survey of biases in the measurement of plasma lipid

and lipoprotein concentrations in 32 lipid clinics in the UK. Ann Clin Biochem 1996;33:82–3.

14. Warnick GR, Wood PD for the National Cholesterol Education Program Working Group on Lipoprotein Measurement. National Cholesterol Education Program recommendations for measurement of high-density lipoprotein cholesterol: executive summary. Clin Chem 1995;41:1427–33.

15. Sugiuchi H, Uji Y, Okabe H, Irie T, Uekama K, Kayahara N, Miyauchi K. Direct Measurement of high-density lipoprotein cholesterol in serum with polyethylene glycol-modified enzymes and sulfated α-cyclodextrin. Clin Chem 1995;41:717–23.

16. Myers GL, Cooper GR, Henderson LO, Hassemer DJ, Kimberly M. Standardization of lipid and lipoprotein measurements. In: Rifai N, Warnick GR, eds. Laboratory measurement of lipids, lipoproteins and apolipoproteins, Washington, DC: AACC Press, 1994:177–205.

17. Vanderlinde RE, Bowers GN, Jr, Schaffer R, Edwards GC. The National Reference System for Cholesterol. Clin Lab Med 1989; 9:89–104.

18. Hainline A, Jr, Karon J, Lippel K (eds.). Manual of laboratory operations: lipid and lipoprotein analysis, 2nd ed. Bethesda, MD: National Heart, Lung and Blood Institute, Lipid Research Clinics Program. HEW Pub. No. NIH 75–628 (rev.). U.S. Government Printing Office Publication No. 1982–361-132:678.

19. Passing H, Bablok W. A new biomedical procedure for testing the equality of measurements from two different analytical methods. J Clin Chem Clin Biochem 1983;21:709–20.

20. NCCLS. Tentative Guideline EP5-T. User evaluation of precision performance of clinical chemistry devices. Villanova, PA: National Committee for Clinical Laboratory Standards, June 1984.