We evaluated the performance of a homogeneous assay for the automated measurement of high-density lipoprotein cholesterol (HDL-C) and compared it with a conventional precipitation technique in the following groups of people: control subjects (group A), clinically-healthy elderly (group B), myocardial infarction patients (group C), nephrotic syndrome patients (group D), and liver cirrhosis patients (group E). The performance of the technique was acceptable with respect to precision, accuracy, linearity, and detection limit. Triglycerides up to 40 mmol/L and bilirubin up to 150 μmol/L did not cause interferences. Hemoglobin decreased HDL-C measurements. Samples were stable at 20 °C for up to four months. Bland–Altman plots showed a good agreement between both techniques in the control group but with a progressive divergence in the patient groups B to E. Results indicate limitations of the technique in certain clinical conditions and, coincidentally, the need for reliable calibration materials.

The monitoring of high-density lipoprotein cholesterol (HDL-C) is clinically important because the concentration of HDL-C in plasma has been well documented as being inversely correlated with the risk of cardiovascular disease (1, 2). HDL-C concentrations <0.9 mmol/L indicate a high risk for cardiovascular disease, and HDL-C ≥1.6 mmol/L is considered protective (3). The National Cholesterol Education Program (NCEP) recommends that HDL-C be measured in all adults at their initial screening (3, 4). Hence, there is an increasing demand in clinical laboratories for reliable and easy-to-perform methods for determining HDL-C.

The reference method for HDL-C measurement is preparative ultracentrifugation (5), which is a cumbersome, time-consuming, and expensive technique. In most clinical laboratories, HDL-C is routinely measured by precipitation methods that are simpler and are in excellent agreement with those of ultracentrifugation (6, 7). However, these methods involve manipulation of the sample (a centrifugation step and recovery of supernatant), which precludes the full automation that is often a prerequisite for a high throughput clinical chemistry laboratory.

Recently, several reports (8–15) have described homogeneous (direct) assays for HDL-C that are readily adaptable to automated analyzers, such that laboratories can have HDL-C determination as an on-line procedure for the routine screening of large populations. However, the application of these assays to specific, well-defined groups of patients has not been sufficiently investigated to date (16), a lack that has considerable importance in terms of efficient patient management.

The present study was aimed to: (a) evaluate a new homogeneous HDL-C assay; and (b) compare this assay with a well-accepted precipitation method in a range of clinically healthy subjects and in the elderly, as well as in patients with cardiovascular disease, nephrotic syndrome, or liver cirrhosis, pathologies in which lipoprotein profiles/concentrations are profoundly altered.

Materials and Methods

All the measurements were done in a ILab® 900 automated analyzer (Instrumentation Laboratories), which facilitates the automated performance of spectrophotometric and turbidimetric reactions at 37 °C, using one or two reagents.
PROCEDURES

Direct HDL-C assay. The direct assay of HDL-C was performed with commercial reagents obtained from Dai-ichi (Tokyo, Japan) and supplied in Spain by ITC Diagnostics (Izasa, Barcelona, Spain). The assay requires two reagents. In the first step, 2 μL of plasma are incubated for 3.5 min with 210 μL of a mixture of polymers and polyanions that block, by adsorption, the apoprotein B-containing lipoprotein particles. In the second step, the noncomplexed cholesterol is measured by the cholesterol oxidase-peroxidase (CHOD-PAP) method.

HDL-C determination by precipitation. HDL-C was measured by precipitation with polyethylene glycol (17). The precipitating reagent was 95 g/L polyethylene glycol (molecular weight, 20 000) dissolved in phosphate buffer (0.033 mol/L NaH2PO4 and 0.066 mol/L Na2HPO4, pH 6.5). In this technique, 500 μL of precipitating reagent is added at 4 °C to 500 μL of plasma. The mixture is vigorously mixed for 15 s and centrifuged at 3000 × g at 4 °C for 20 min to pellet the apoprotein B-containing lipoproteins. HDL-C was measured in the supernatant by the CHOD-PAP technique. The intra- and interassay CV values were <3% and <5%, respectively. In a preliminary assessment, we observed that the values obtained by this technique did not differ significantly from those obtained by sequential preparative ultracentrifugation in control subjects (r = 0.90; y = 1.1x − 0.1; n = 30) nor in cirrhotic patients (r = 0.89; y = 0.9x; n = 37).

Other methods. Cholesterol and triglycerides were determined enzymatically with the CHOD-PAP and the lipase/GPO/PAP methods, respectively; albumin was determined by the bromcresol green method; bilirubin was determined by the Jendrassik and Gröf technique (ITC Diagnostics, Barcelona, Spain); IgG and IgM were determined by immunoturbidimetry (Biokit, Barcelona, Spain); hemoglobin was determined in a Coulter® MD-10 counter (Coulter).

STANDARD AND CONTROL MATERIALS

Initially, two commercial HDL-C calibrators, one from ITC Diagnostics and the other from Sigma Diagnostics® (EZ HDL™ calibrator, Sigma Chemicals) were evaluated against purified HDL obtained by ultracentrifugation (18). The calibrator from ITC Dignostics gave cholesterol values ∼20% higher than those reported by the manufacturers, thus undervaluating HDL-C results when used as a standard. Conversely, the cholesterol value of the Sigma calibrator was identical to that reported in the commercial leaflets; for this reason, the Sigma calibrator was the standard used in the present evaluation. Lipid-Trol-TL Control Levels 1, 2, and 3 (Dade®, Düdingen, Switzerland) and three pools of sera (concentrations established by direct method) designated as “low” (0.61 ± 0.01 mmol/L), “normal” (1.13 ± 0.01 mmol/L), and “high” (1.99 ± 0.02 mmol/L) were used as quality controls.

SAMPLES

Blood samples were selected from 98 control subjects (group A; ages, 24–55 years) and from 240 assorted other individuals: 85 elderly who were clinically healthy (group B; ages, 72–93 years); 93 patients who had had at least one episode of acute myocardial infarction (AMI) before the age of 55 years (group C; actual ages, 57–65 years); 25 patients presenting with a nephrotic syndrome (group D; ages, 26–64 years); and 37 patients with liver cirrhosis (group E; ages, 45–71 years). To preclude hormonal status effects on the measurements, only blood samples from men were used in the present study.

The control subjects were randomly chosen from the routine health and safety-at-work checks conducted in several industrial companies in our area. Excluded were those with clinical or laboratory evidence of diabetes, neoplasia, renal disease, hepatic damage, and cardiovascular disease. Group B subject samples were from routine health monitoring in several residences for the elderly in the area. Patient samples for groups C, D, and E were from the outpatient clinics of the Hospital Universitari de Sant Joan de Reus. All procedures were in accordance with the ethical standards of the Ethics Committee of Hospital Universitari de Sant Joan, and anonymity of results was guaranteed. All blood samples were drawn in the fasted state into EDTA-containing glass tubes; the plasma was separated by centrifugation at 1500 × g for 15 min and stored at −20 °C for batched analysis.

PERFORMANCE EVALUATION OF THE DIRECT HDL-C MEASUREMENT

The direct HDL-C assay was evaluated as described elsewhere (19).

STATISTICAL ANALYSIS

Mean values for HDL-C by the two methods were compared by Student’s t-test. Statistically significant differences were set at P < 0.05. Results are presented as means ± SD. The association between variables was measured by linear regression analysis. The degree of agreement between the direct and the precipitation methods was estimated by the Bland–Altman graphical procedure (20) as described by Hollis (21). In this method, a scattergram is drawn with the means of the results obtained by the two procedures plotted against the differences between them. Bias is noted as a lack of symmetry of these differences around the value zero, and the limits of agreement are given by the mean difference ± 2 SD of the differences.

Results

PERFORMANCE EVALUATION OF THE DIRECT HDL-C MEASUREMENT

Precision. Intraassay precision was determined with 20 replicate analyses of the three Lipid-Trol-TL controls. To assess interassay precision, aliquots of these controls
stored at −20 °C were analyzed over 20 consecutive days. The CV values are shown in Table 1.

**Total error.** Total error was calculated by adding the systematic error and the random error, as previously described (22). Results were as follows: 11.6% for Lipid-Trol-TL Control Level 1; 11.3% for Level 2, and 6.5% for Level 3.

**Accuracy.** Accuracy was estimated by adding 100 μL of normal pool serum to identical volumes of the three commercial controls. The percentage recoveries (calculated as measured HDL-C/theoretical HDL-C × 100) from sextuplicate measurements were 98.7% ± 2.0% for Level 1, 104.0% ± 4.6% for Level 2, and 101.7% ± 1.6% for Level 3.

**Linearity and detection limit.** Linearity was assessed by quintuplicate measurements on serial dilutions of the high pool (from undiluted to up a 1:32 dilution with physiologic saline). The regression line of observed vs expected values was \( y = 0.99x + 0.04 \) (\( r = 0.9995 \)). To determine the detection limit, the absorbance of the reagent blank was measured 20 times, the mean ± SD calculated, and the detection limit defined as the HDL-C concentration corresponding to an absorbance equal to the mean of the reagent blank value + 2 SD. The detection limit thus calculated was 0.10 mmol/L.

**Interferences.** Assessment of interference from triglycerides, hemoglobin, and bilirubin was performed as previously described (23). The low, normal, and high sera pools were supplemented with chylomicrons, hemoglobin, or bilirubin at various concentrations. Results are shown in Fig. 1. There was no substantial interference from triglycerides up to 40 mmol/L nor from bilirubin up to 150 μmol/L, but higher bilirubin concentrations produced a marked decrease in the direct HDL-C measurement. Hemoglobin interference strongly decreased the HDL-C value and would suggest that the assay is invalid for hemolyzed samples.

**Effect of paraproteinemia.** The effect of nonspecific paraproteinemia in HDL-C determination was assessed as the percentage recovery after the addition to the low pool of three serial dilutions of sera from two patients with IgG (47.9 g/L) and IgM (17.6 g/L) myeloma. Results are shown in Table 2. The recovery was not influenced by the paraprotein concentration.

**Stability study.** Aliquots of the low, normal, and high pools stored at −20 °C were periodically thawed over 4 months. The HDL-C values did not vary significantly (\( P > 0.05 \)) over this period (Fig. 2).

**Comparison between direct and precipitation methods**

Values for HDL-C obtained by the precipitation and by the homogeneous assays are shown in Table 3. Results were practically identical in the samples from control subjects and from the elderly. The homogeneous assay gave results somewhat lower in samples from patients with AMI (difference between means, −2.8%). However, these differences did not reach statistical significance. Differences between means were higher and statistically significant in samples from patients with the nephrotic syndrome (−7.4%; \( P < 0.05 \)) and were particularly high in the samples from patients with liver cirrhosis (−20.8%, \( P < 0.001 \)). Pearson correlation coefficients comparing the two methods were highly significant (\( P < 0.001 \); Figs. 3 and 4, top panels). Tighter regressions were observed for control and elderly groups (\( r = 0.90 \) and 0.95, respectively) than for those of the AMI, nephrotic, and cirrhotic patient groups (\( r = 0.76, 0.69, \) and 0.70, respectively).

However, because correlation coefficients are measures of the association between two methods but not of the agreement between them (21), the degree of agreement was assessed using the Bland–Altman (20) graphical technique (Figs. 3 and 4, bottom panels). These graphs are the differences between the two methods (y-axis) plotted against the average between them (x-axis). Agreement is indicated by the calculated value of the bias derived from the mean and SD of the differences.

For samples from control subjects, the mean difference was −0.01 mmol/L, which indicated that the homogeneous assay was in concordance with the precipitation method and did not systematically under- or overestimate HDL-C values. From this group, four samples (4.1%) had values >2 SD above or below the mean, i.e., outside the limits of agreement (21). The same plot for samples from the elderly group is shown in Fig. 3 (bottom panel, right). As with the samples from the control subjects, the mean difference was also

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**Table 1. Analytical precision of homogeneous HDL-C.**

<table>
<thead>
<tr>
<th>Control material</th>
<th>Mean HDL-C, mmol/L</th>
<th>SD, mmol/L</th>
<th>CV, %</th>
<th>SD, mmol/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1</td>
<td>0.73</td>
<td>0.02</td>
<td>2.7</td>
<td>0.05</td>
<td>7.1</td>
</tr>
<tr>
<td>Level 2</td>
<td>0.95</td>
<td>0.03</td>
<td>3.1</td>
<td>0.04</td>
<td>4.0</td>
</tr>
<tr>
<td>Level 3</td>
<td>1.56</td>
<td>0.04</td>
<td>2.6</td>
<td>0.07</td>
<td>4.5</td>
</tr>
</tbody>
</table>

* Aliquots of Lipid-Trol-TL control materials (Levels 1, 2, and 3) were kept at −20 °C until analyzed (n = 20).
Fig. 1. Interference of triglycerides, bilirubin, and hemoglobin with the homogeneous assay.

(A) Chylomicrons added to 40 mmol/L; (B) bilirubin added to 495 μmol/L; (C) free hemoglobin added to 5 g/L.
close to zero (−0.01 mmol/L), but the differences would seem to be dependent on the HDL-C concentration because there appeared to be an overestimation by the homogeneous technique at low HDL-C concentrations and an underestimation at values >1.5 mmol/L. Two samples (2.3%) were outliers with discrepancies >2 SD above or below the mean.

The degree of agreement between both techniques was worse in the other groups of patients (Fig. 4, bottom panels). In the samples from subjects who had experienced an AMI (left panel), the mean difference was close to zero (−0.03 mmol/L), but the limits of agreement were very high, i.e., values showed a considerable degree of dispersion above or below the mean. In the nephrotic syndrome samples (middle panel), the degree of disagreement was similar (mean difference, −0.09 mmol/L). The worst results were observed in samples from patients with liver cirrhosis, in whom a clear underestimation was noted when the homogeneous assay was used. The mean difference was very low (−0.26 mmol/L), as were the limits of agreement (from −1.04 to 0.50 mmol/L).

Interestingly, the disagreement between the homogeneous and the precipitation techniques in samples from patients with liver cirrhosis seems to be associated with the degree of liver damage. As seen in Fig. 5, bias plots show that the differences between the methods depend on serum albumin and bilirubin concentrations, used in this evaluation as markers of liver-function impairment.

**Discussion**

In our experience, the precision of this assay was similar to that of conventional precipitation methods (24) but lower than those previously published for the Daiichi reagent (13) or other homogeneous assays (13-15, 22). The NCEP performance goals for 1998 demand that the CV of

| Table 2. Effect of paraproteinemia on direct HDL-C measurement. |
|-----------------------|-----------------------|-----------------------|
| Recovery, %a | IgG (47.9 g/L) | IgM (17.6 g/L) |
| Dilution | | | |
| 1:2 | 99 | 101 |
| 1:4 | 100 | 100 |
| 1:8 | 103 | 105 |

a Percentages of recovery after adding serial dilutions of sera from two patients with IgG and IgM myeloma to aliquots of the low pool.

<table>
<thead>
<tr>
<th>Table 3. Assay results (mmol/L).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
</tr>
<tr>
<td>Triglycerides</td>
</tr>
<tr>
<td>HDL-C (precipitation)</td>
</tr>
<tr>
<td>HDL-C (homogeneous)</td>
</tr>
<tr>
<td>Difference means,f %</td>
</tr>
</tbody>
</table>

a P < 0.05 with respect to group A.
b P < 0.001 with respect to group A.
c P < 0.01 with respect to group A.
d P < 0.05 with respect to HDL-C (precipitation).
e P < 0.001 with respect to HDL-C (precipitation).
f Differences between the means of HDL-C obtained by precipitation and by the homogeneous assay.
HDL-C determinations be <4% at concentrations >420 mg/L (1.09 mmol/L) and that the SD be <17 mg/L (0.04 mmol/L) at concentrations <420 mg/L (25). These criteria were met by our intraassay CVs and by the interassay CV for Lipid-Trol Level 2 but were slightly exceeded in the interassay CVs for Levels 1 and 3. It is doubtful if such marginal differences would greatly impair clinical judgment.

At the three HDL-C concentrations studied, the assay fulfilled the NCEP total-error goal for 1998 of <13% (25). Our results differed those of Huang et al. (13), who, also using a Daiichi reagent, found very high total errors. A possible explanation for this discrepancy is the high systematic error they obtained when using the original calibrator material supplied by the manufacturers. Indeed, our results showed that the cholesterol concentration reported for this calibrator was too low and gave values that were 20% higher when assayed against purified HDL. This systematic error was resolved when a different standard material was used. These results would suggest that the accurate preparation of HDL-C standards could be a general problem that manufacturers of these particular materials ought to seriously address.

Accuracy and linearity were excellent. The detection limit was 0.10 mmol/L, which is sufficient to measure HDL-C at low concentrations reliably.

Lipemia up to a triglyceride concentration of 40 mmol/L appeared to have little influence on the assay. Bilirubin up to 150 μmol/L had no influence on the accuracy, but higher values induced a negative interference. This effect has been reported for other HDL-C assays (11, 13, 22) and would seem to be a function of cholesterol measurements in general (26). Paraproteins did not cause any interference with the assay. Hemoglobin markedly decreased the values obtained for HDL-C, which is surprising because this effect has not been reported for other, equivalent homogeneous assays (11, 13). Although hemoglobin has been shown to interfere with enzymatic cholesterol determinations (26), the presence of free hemoglobin could lead in the present assay system to alterations in matrix formation, which is the basis of the direct assay. Again, we differ from Huang et al. (13), who described a different interference pattern of the Daiichi reagent (negative interferences by bilirubin and triglyceride but not by hemoglobin). We cannot offer any explanation for this discrepancy except to suggest,
perhaps, a qualitative modification in the manufacture of the reagent subsequent to that article.

Storage of samples at \(-20 \, ^\circ\text{C}\) for up to 4 months had no effect on the measurements of HDL-C and is an advantage for retrospective clinical or pathophysiological investigations.

The method comparison studies showed a good agreement between the homogeneous assay and the precipitation technique in the control group. For example, there was a high correlation between both techniques \((r = 0.90)\), and the regression line was very close to identity \((\text{slope} = 0.96; \text{intercept} = 0.03 \, \text{mmol/L})\). This correlation was somewhat lower than those reported in other evaluations; ranging from 0.95 and 0.99 \((13, 14)\). Direct comparisons are not acceptable because these studies were performed on randomly obtained samples and the present study was conducted on samples from a control group of clinically healthy individuals who would not necessarily exhibit a wide range of HDL-C values. In addition, the Bland–Altman graphical assessment of agreement was good because there was no evidence of bias \((\text{the mean difference was very close to zero; } -0.01 \, \text{mmol/L})\) and the limits of agreement were narrow; both parameters indicate good concordance between the two methods of measurement.

With respect to samples from patients with certain pathologies, however, a completely different situation prevailed. In the samples from the elderly, ostensibly healthy subjects, the correlation between both methods was still high \((r = 0.95)\), but there was an obvious inverse relationship between the differences and the average of HDL-C concentrations (Fig. 3, bottom right panel). In the samples from patients who had evidence of premature atherosclerosis \((\text{AMI } < 55 \, \text{years of age; group C})\) and the nephrotic syndrome patients \((\text{group D})\), there was a lack of correlation between the two methods, as demonstrated by the lower correlation coefficients as well as the broader limits of agreement in Bland–Altman plots. The worst concordance was observed in the samples from patients with liver cirrhosis \((\text{group E})\). In these samples, the homogeneous method underestimated HDL-C concentration, and the differences between the methods were clearly dependent on the concentrations being measured: values of up to \(-2.0 \, \text{mmol/L}\) when the average concentration being measured was \(2.5 \, \text{mmol/L}\). Of considerable interest was that the differences between the methods were related to serum bilirubin and albumin concentrations; two biochemical markers indicative of liver impairment.

This lack of agreement between the two methods in the samples from patients with certain pathologies is, perhaps, of crucial importance. The disagreement was within acceptable limits in the samples from the elderly group but progressively worsened in the samples of subjects with AMI and the nephrotic patients and produced very

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**Fig. 4.** Correlations (top panels) and Bland–Altman plots (bottom panels) for HDL-C in (A) patients with myocardial infarction, (B) patients with nephrotic syndrome, and (C) patients with cirrhosis.

The correlation equations for the upper panels are as follows: (A) \(y = 0.90x + 0.06, r = 0.76, P < 0.001\); (B) \(y = 0.92x + 0.01, r = 0.69, P < 0.001\); (C), \(y = 0.47x + 0.40, r = 0.69, P < 0.001\). In the top panels, the dashed lines represent the lines of identity.
aberrant results in samples from patients with cirrhosis. The differences between the methods were not related to HDL-C or to the total cholesterol or triglyceride concentrations being measured; hence, a physiological explanation is difficult to propose. The only group in which an interpretation may be tentatively hypothesized is that of the cirrhotic patients. These subjects present with major alterations in HDL lipid and protein composition (27, 28), and perhaps more importantly, with structural alterations in the HDL particle, often exhibiting flat, discoidal shapes (termed “rouleaux”) in the circulation and that tend to form aggregates in vitro (29). The possible interference of these alterations on the physico-chemical basis of the separation of the various lipoprotein classes on which the homogeneous HDL-C measurement depends is beyond the scope of the present investigation. However, the significant association between the method variation and the degree of liver dysfunction would lend credence to the suggestion that progressive physical alterations of the lipoproteins would produce progressive inaccuracy in the HDL-C measurement.

In conclusion, the present study demonstrates that, with respect to the parameters of method comparison, the direct method for measuring HDL-C is effective, inexpensive, and readily automated and lends itself to the screen-
ing of large populations. The limitations of the technique are highlighted in individual groups of patients in whom gross alterations in lipoproteins are encountered.

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