enzymatic assay for ethylene glycol, however, has not been previously documented. The interference may not have been appreciated in previous evaluations of the assay because test compounds, such as lactate and alcohols, were added to normal serum, not sera from critically ill patients [2]. To evaluate the performance of the enzymatic assays for ethylene glycol and ethanol in hospitalized patients with abnormal serum chemistry, we added D1-lactic acid to two random serum samples with markedly increased LD and performed the enzymatic assays (Table 1). Lactate per se does not interfere in the assay; however, the concomitant presence of LD under the proper assay conditions produces NADH, resulting in substantial false-positive values in the enzymatic assays for ethylene glycol and ethanol. Given their correlation with the concentrations of LD and lactate acid, these false-positive values may be due to the LD-catalyzed conversion of serum lactate and reagent NAD⁺ to pyruvate and NADH.

To support this hypothesis, we reconstituted normal serum with increasing concentrations of LD-5 purified from human placenta (Sigma Chemical Co., St. Louis, MO) in the presence of 0, 10, or 50 mmol/L D1-lactic acid (Sigma). The ethylene glycol enzymatic assay gave falsely positive results when LD serum activity was >3000 U/L, as did the ethanol enzymatic assay when LD serum activity was >2000 U/L (Fig. 1). The interference was most pronounced when the concentration of D1-lactate exceeded 10 mmol/L. These data, however, should not be strictly extrapolated to the clinical setting because the contribution to total LD activity in this experiment was due solely to LD-5, and the concentration of lactate consisted of approximately equal amounts of L- and D-isomers. In hospitalized patients, increased LD activity may be due primarily to the contribution of a different isoenzyme or of more than one isoenzyme, and increased lactate concentration will most probably be the L-isomer. However, the data obtained from three hospitalized patients with increased serum LD activity (Table 1) support a clinically relevant guideline that significant false-positive results occur when the LD serum activity is at least 12-fold greater than the upper limit of the reference interval, and lactate is concomitantly at least 10-fold greater than the upper limit of its reference interval.

High concentrations of lactate and LD in sera from critically ill patients interfered in the enzymatic assay for ethylene glycol by increasing the production of NADH. Although lactate added to normal sera does not interfere with the assay, results should be interpreted cautiously in samples with high LD and lactate concentrations. Other interferences reported for the ethylene glycol enzymatic assay include glyceraldehyde and glyceral, which can be oxidized by the enzyme glyceral dehydrogenase [2]. The enzymatic assay is otherwise very specific, and other alcohols—including ethanol, methanol, and isopropanol—do not interfere with interpretation. Ethylene glycol poisoning of previously healthy individuals may result in lactic acidosis but will not typically result in increased LD. However, this analytical interference becomes clinically relevant in cases of suspected ethylene glycol poisoning of individuals with hepatic, renal, or cardiac disease who may manifest both lactic acidosis and increased serum LD. Positive results in the enzymatic assay for ethylene glycol in such cases should be confirmed with a different method such as gas chromatography.

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References


According to the recommendations of the National Cholesterol Education Program Adult Treatment Panel, low-density lipoprotein cholesterol (LDL-chol) should be used for screening and as a primary treatment criterion for patients with increased total cholesterol concentrations [1]. This makes the need for accurate measurements of LDL-chol a national public health imperative. The use of the Friedewald equation is attractive, but its accuracy is in doubt in plasma samples with triglyceride (TG) concentrations >4.5 mmol/L (400 mg/dL) [2-7]. A decennium ago we found the Friedewald equation to be accurate up to a TG concentration of 8 mmol/L [8]. Assuming that similar plasma samples were analyzed, these findings raise questions about the quality of the methods used in other laboratories, especially in the analysis of hypertriglyceridemic samples. The disposal of a direct LDL-chol method not interfered with by hypertriglyceridemia [9] prompted us to evaluate its accuracy in hyperlipidemic samples, including a selection with familial dysbetalipoproteinemia (FD). We hoped that this revaluation could shed more light on our previously reported claim concerning the accuracy of the Friedewald calculation compared with that of the reference method [8].

Overnight fasted blood samples from healthy persons and from patients with various types of hyperlipoproteinemia were drawn into Vacutainer® Tubes containing EDTA (Becton Dickinson, Meylan Cedex, France). Plasma samples were analyzed fresh. If sufficient amounts of plasma were available, two aliquots were stored at -80°C for >3 months; one was supplied with saccharose (final concentration 6 g/L). Patients were classified into the different phenotypes according to the criteria of Fredrickson et al. [10] with cutoff limits for plasma cholesterol, TG, and LDL-chol of 6.5, 2.0, and 4.6 mmol/L, respectively. Plasma samples with lipemia, especially those from subjects with FD, were preferentially included. This means that the frequency of samples with FD was considerably higher than in the healthy population. The 217 fresh plasma samples analyzed included, by selection, 63 with a plasma TG concentration >4.5 mmol/L, among which were 31 with a plasma TG >8.0 mmol/L; 11 had a VLDL-chol/plasma TG ratio >0.69.
(diagnostic for FD [11]). The latter subjects were homozygotes for apoprotein E2 [12]. Following the strategy of our preceding study [9], we classified plasma of subjects with fasting plasma TG concentrations <8 mmol/L as phenotype IV and those >8 mmol/L as phenotype V hyperlipoproteinemia.

Besides the 217 samples analyzed fresh with the ultracentrifugation method and the Friedewald formula, 147 fresh samples were also available for analysis with the immunoseparation method; 110 of these samples could be analyzed both fresh and after freeze-thawing, including 64 to which secaehrose (final concentration 6 g/L) was added before freezing.

The reference method we used was a combined ultracentrifugation/prefractionation procedure. VLDL-cholesterol was determined directly in the VLDL isolated by sequential ultracentrifugation [13]. After ultracentrifugation of plasma in the TFE-45.6 rotor (Kontron, Zürich, Switzerland) for 16 h at 131 000 g in the Beckman L7-55 ultracentrifuge at 14 °C, the density <1.006 kg/L fraction was aspirated by means of a rubber bulb Pasteur pipette. LDL-cholesterol was determined in plasma after precipitation of the VLDL and LDL with phosphotungstic acid and MgCl₂ (cat. no. 543004; Boehringer Mannheim, Mannheim, Germany). LDL-cholesterol was calculated by subtraction. Reproducibility, expressed as the CV, was 4.3% for VLDL-cholesterol determination, 2.6% for HDL-cholesterol determination, and 2.3% for LDL-cholesterol calculation (n = 30).

The LDL-immunoseparation reagent was obtained from Sigma (Sigma LDL-cholesterol, cat. no. 353-A, lot no. 05516275; St. Louis, MO). The procedure was performed according to the manufacturer's directions. The LDL-cholesterol reagent consists of a suspension of polystyrene latex beads coated with goat polyclonal antibodies to human apoproteins in a buffer containing 1 g/L sodium azide. From this reagent 200 µL is pipetted into the inner compartment of a separation tube (fitted microcentrifugation tubes); to the same compartment we added 30 µL of either the controls (concentrations low and high, included in the kit) or plasma, capped the tubes, and vortex-mixed them. After incubation for 5 to 10 min at room temperature, we centrifuged the tubes for 10 min at 4300 g at room temperature. Subsequently, the filtrate in the outer compartment was vortex-mixed and assayed for cholesterol. Although the vortex-mixing step was not mentioned in the prescription of the supplier, it was found to be essential for obtaining precise results. The cholesterol concentration determined in the filtrate was multiplied by 7.35 to obtain the LDL-cholesterol concentration in plasma. The precision, as expressed by the interassay CV, was 5.8% and 4.9% for the low- and high-concentration control, respectively (n = 10).

As mentioned before [8], the accuracy of the Friedewald formula (LDL-cholesterol = total cholesterol - HDL-cholesterol - 0.45 × plasma triglycerides) is influenced by the accuracy of the methods used for the determination of cholesterol, TG, and HDL-cholesterol. Furthermore, the relation between the concentration of plasma total TG and VLDL-cholesterol is dependent on age, sex, and sociodemographic variations of the subjects studied [14]. For most accurate results we used a value of 0.42 × TG as the best approach for VLDL-cholesterol [8].

Cholesterol was determined (with the reagent of Boehringer Mannheim, cat. no. 1489704) on the Hitachi 747 analyzer. For samples containing HDL, a micro method was used, in which 20 µL of sample was mixed with 250 µL of reagent for 10 min. Other conditions were similar to the macro procedure applied for total plasma cholesterol. We used a calibrating serum for automated systems (Boehringer Mannheim cat. no. 759350). The accuracy of the procedure was checked against the improved Abell–Kendall procedure and the bias was <0.3%; imprecision was 1.5% (n = 30). TG were analyzed on the Hitachi 747; the accuracy was checked against a semi-automated colorimetric method [15]; imprecision was 1.6% (n = 30) for concentrations up to 12 mmol/L. The accuracy of the HDL-cholesterol method was confirmed by comparison with sequential ultracentrifugation and against the polyethylene glycol-6000 precipitation method [16, 17] and unpublished observations). The HDL-method used appeared to be very convenient for normo- and hyperlipidemic plasma in that very few samples had to be ultracentrifuged to clear turbid supernatants.

Results were analyzed by Student's paired t-test. Results obtained by different methods were correlated by using Pearson's correlation test with the application of the SSPI/PC statistical software (version 3.1) (SSPS, Chicago, IL). The standard errors in the intercept, slope, and estimate (S₀', S₀, respectively) were also calculated by using the test of Passing and Bablock [8]. For all intermethod comparisons the deviations in intercept and slope did not deviate from the ideal curve y = x. Differences were considered significant at P < 0.05.

We applied the various methods for determining LDL-cholesterol in 217 fresh plasma samples with TG concentrations ranging from 0.41 to 50.1 mmol/L, including 31 samples with a concentration >8.0 mmol/L and 11 samples of subjects with obligate FD with VLDL-cholesterol/TG ratio >0.69. The results obtained with the LDL direct method agreed better with the reference method than the estimated LDL-cholesterol values (correlation coefficients 0.94 vs 0.85, S₀' values 0.34 vs 0.55, respectively). However, after exclusion of the samples with plasma TG >8.0 mmol/L and the samples of the patients with FD (all having a VLDL-cholesterol/plasma TG ratio >0.69), the results (n = 177) obtained with the Friedewald equation correlated very well with the reference method, similarly as those obtained with the LDL-direct method (n = 115) (correlation coefficients >0.97, S₀' values <0.21, regression equations by Passing and Bablock analysis different from y = x). These results are in agreement with those reported previously [8]. Results obtained for the non-FD plasma samples with TG concentrations between 4 and 8 mmol/L were explicitly similar to the general mean results (data not shown). These findings can be explained by the relative constancy of the ratio VLDL-cholesterol/plasma TG in the plasma samples with TG concentrations up to 14 mmol/L (Fig. 1). Thus, we did not obtain evidence for a shift in the chemical composition data as a result of an excess of chylomicrons at increasing plasma TG values.

In the non-FD samples with TG <8.0 mmol/L, in which the Friedewald approach gave accurate values, intermethod precision of the Friedewald–ultracentrifugation comparison and the direct LDL-cholesterol comparison were similar (S₀' values 0.17 vs 0.21, respectively).

A negative bias of ~11% was obtained with the direct method (y) after prior storage of the plasma samples at −80 °C compared with the reference method (x): mean x, 4.12 ± 1.98 mmol/L; mean y, 3.58 ± 1.75 mmol/L; linear regression equation y = 0.88x − 0.02, correlation coefficient r = 0.93, S₀' = 0.35, n = 110. Apparently, some of the LDL was retained in the column. Addition of 6 g of saccharose per liter of plasma before freezing prevented this, as could be derived from the statistical results: mean x, 4.01 ± 1.98 mmol/L; mean y, 3.81 ±
The drawing of conclusions concerning the optimal estimation we observed, independent of the plasma TG concentration. Our Friedewald formula is biased or imprecise, especially when observations suggest that in other laboratories one of the TG (in mmol/L) as an approach. However, even in the non-FD analysis, data not shown). This casts doubts on the suggestion that the analysis of fasting plasma samples in our study merely the reference value did not differ from 1.0 (Passing and Bablock et al. [20]).

Up to a TG concentration of 8 mmol/L the Friedewald approach and the direct LDL method gave similar scores both for accuracy and precision, stressing the validity of our experiments in general and the accuracy of our reference method especially. The greater applicability of the Friedewald calculation in our hands could be due to the fact that we analyzed fasted plasma samples. Chylomicrons present in nonfasting plasma are thought to result in too low LDL-chol values because VLDL-chol in these samples is overestimated when using 0.45 × plasma TG (in mmol/L) as an approach. However, even in the non-FD samples with a plasma TG concentration of 8 to 14 mmol/L, characterized by increased concentrations of chylomicrons, statistical analysis revealed that the intercept of the curve depicting the relation between the estimated and determined LDL-chol vs the reference value did not differ from 1.0 (Passing and Bablock analysis, data not shown). This cast doubts on the suggestion that the analysis of fasting plasma samples in our study merely explains the difference in the applicability of the Friedewald formula compared with other laboratories. This is also supported by the relatively constant estimate for VLDL-chol that we observed, independent of the plasma TG concentration. Our observations suggest that in other laboratories one of the analytical methods delivering values for substitution in the Friedewald formula is biased or imprecise, especially when hypertriglyceridemic plasma samples are analyzed. This is supported by the results of McNamara et al. [2]: At TG concentrations >4.00 g/L (4.5 mmol/L), large standard deviations prevent the drawing of conclusions concerning the optimal estimation factor (TG/4.5 to TG/8) as an approach for VLDL-chol, in contrast to our results. We can only speculate which of the methods is responsible for the reported large variation of the bias between estimated and measured LDL-chol values. It is generally known that HDL-chol analysis in hypertriglyceridemic plasma samples is potentially biased because of possible incomplete removal of the apo B-containing lipoproteins [19]. Theoretically, also, the method for the determination of TG may be biased in hypertriglyceridemic samples. Standardization on the basis of the CDC protocol does not exclude this, because only normo- or slightly hyperlipidemic controls have to be analyzed in this program. Because of the lack of suitable control material, the quality of the analyses at higher degrees of lipemia is at present uncertain. This is more true in methods requiring lipemic samples to be severalfold diluted for proper calibration. It is also possible, as already suggested by Friedewald et al. [20], that the reference method is biased or imprecise when hypertriglyceridemic plasma samples are analyzed. To prevent this we determined VLDL-chol directly, whereas most other laboratories determine VLDL-chol indirectly as the difference of total cholesterol and the cholesterol present in the d >1.006 kg/L fraction. The latter procedure can result in highly variable VLDL-chol concentrations involving imprecise cholesterol analysis. In the same way, relatively large CVs of the methods used in these evaluations overestimate the bias in LDL-chol of the Friedewald approach, especially for lipemic plasma. In plasma samples with TG >8.0 mmol/L the direct method is preferred over the Friedewald approach. Until now, large-scale use of the direct method is prevented by the instability of the analyte when stored frozen [20]. Irrespective of the value of any method in the risk estimation for coronary heart disease, the support of a lipid reference laboratory remains necessary because of the limited value of both the Friedewald approach and the direct LDL-method for proper phenotyping of strongly hyperlipidemic samples. Considering the fact that the measured plasma triglyceride concentration also gives insight into the LDL subfraction pattern [21, 22] and in the presence of dense LDL, reportedly being associated with increased atherogenesis [23, 24], the use of the Friedewald formula is recommended up to a TG concentration of 8 mmol/L.

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References


