

Analytical performance of a direct assay for LDL-cholesterol: a comparative assessment versus Friedewald's formula

LDL kolesterol tayini için direkt yöntemin analitik performansı: Friedewald formülü ile kıyaslanması

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ABSTRACT

Objective: Because low density lipoprotein-cholesterol (LDL-C) is a modifiable risk factor for coronary artery disease (CAD), its routine measurement is recommended in the evaluation and management of hypercholesterolemia. Concentrations of LDL-C are commonly monitored by means of the Friedewald formula (FF), which provides a relative estimation of LDL-C concentration when the triglyceride (TGs) concentration is <200 mgr/dl and there are no abnormal lipids. Because of the limitations of the Friedewald calculation, direct methods for an accurate quantification of LDL-C are needed.

Methods: We critically examined an immunoseparation method for direct assay of LDL-C in a comparison with FF. 1) We measured intraassay and interassay precision using quality-control sera and patient serum pools. Accuracy was evaluated from total error analyses. Sample stability was examined over 2 months. 2) The LDL-C levels obtained with direct assay were compared with those calculated by the FF in 47 randomly chosen patient samples. The samples were classified as group 1 (patients with TGs 60-308 mg/dl n=25) and group 2 (patients with TGs 320-695 mg/dl, n=22).

Results: The direct immunoseparation assay displayed an excellent precision (total coefficient of variance (CV) <2.5%, intraassay CV<1.5% and interassay CV<1.5%). Mean total error was 4.34%. The direct assay met the current National Cholesterol Education Program (NCEP) requirements for LDL-C testing for precision and accuracy. The results of direct method (x) and the FF (y) were highly correlated ($r=0.9908$, $y=1.030x-0.289$, $n=25$) in group 1, but the results of two methods disagreed ($r=0.716$, $y=0.956x-24.869$, $n=22$) in group 2 (patients with TGs 320-695 mg/dl).

Conclusion: The direct immunoseparation assay meets the currently established analytical performance goals and may be useful for the diagnosis and management of hyperlipidemic patients. (*Anadolu Kardiyol Derg 2005; 5: 13-7*)

Key words: LDL--Cholesterol; direct assay; Friedewald formula; analytical performance

ÖZET

Amaç: Düşük yoğunluklu lipoprotein-kolesterol (LDL-C) koroner kalp hastalığı (KKH) için değiştirilebilir bir risk faktörü olduğundan, rutin ölçümü hiperkolesteroleminin değerlendirilmesinde ve kontrolünde önerilmektedir. Klinik laboratuvarlarda LDL-C genellikle Friedewald formülü (FF) ile hesaplanır; FF trigliserid konsantrasyonu < 200 mg/dl olduğunda LDL-C konsantrasyonunun rölatif bir ölçümünü verir. Friedewald hesaplamasının bazı limitasyonlarından dolayı LDL-C tayini için daha kesin ve direkt yöntemlere ihtiyaç vardır.

Yöntemler: Bu çalışmada LDL-C tayini için direkt bir immunoseparasyon yöntemini FF ile kıyaslamalı olarak araştırdık. 1) Kalite-kontrol serumları ve hasta serum havuzları kullanarak "intraassay" ve "interassay" presizyonu inceledik. Doğruluk total hata analizleri ile değerlendirildi. Örnek stabilitesi 2 aylık bir süreçte incelendi. 2) Direkt yöntemle elde edilen LDL-C düzeyleri 47 hasta serumunda FF ile hesaplanan sonuçlarla kıyaslandı. Hasta örnekleri grup 1 (TGs 60-308 mg/dl, n=25) ve grup 2 (TGs 320-695 mg/dl, n=22) olmak üzere sınıflandırıldı.

Bulgular: Direkt immunoseparasyon yöntemi mükemmel bir presizyon gösterdi (total (CV) <2.5%, "intraassay" CV<1.5% ve "interassay" CV<1.5%). Ortalama total hata 4.34% idi. Direkt yöntem LDL-C ölçümü için NCEP'nin önerdiği presizyon ve doğruluk kriterlerine uymaktaydı. Direkt yöntemin sonuçları (x) grup 1'de FF ile (y) oldukça uyumluydu ($r = 0.9908$, $y = 1.030x - 0.289$, $n=25$), fakat grup 2'de (TGs 320-695 mg/dl olan olgular) iki yöntemin sonuçları uyumsuzdu ($r = 0.716$, $y = 0.956x - 24.869$, $n=22$).

Sonuç: Direkt immunoseparasyon yöntemi LDL-C için son zamanlarda kabul edilen analitik performans hedeflerini karşılar ve hiperlipidemik hastaların tanı ve izlenmesinde yararlı olabilir. (*Anadolu Kardiyol Derg 2005; 5: 13-7*)

Anahtar kelimeler: LDL-kolesterol, direkt yöntem, Friedewald formülü, analitik performans

Introduction

Coronary artery disease (CAD) is one of the leading causes of death in the world. Cigarette smoking, high blood pressure and increased low-density lipoprotein cholesterol (LDL-C) concentra-

tion are among the strongest causative risk factors for this disease (1,2). According to the National Cholesterol Education Program-Adult Treatment Panel II (NCEP-ATP II), the diagnosis and management of patients with hypercholesterolemia are largely based on the concentration of LDL-C (3). The NCEP-ATP II defines

LDL-C values below 3.37 mmol/l (130 mg/dl) as "desirable" and those over 4.14 mmol/l (160 mg/dl) as "high". In patients suffering from CAD, the tentative treatment goal is to lower LDL-C to 2.6 mmol/l (100 mg/dl) or below(4).

The recently updated NCEP-ATP III guideline, which provides a comprehensive overview of clinical evidence, maintain the focus of diagnosis and treatment efforts on total cholesterol (TC) and LDL-C, with more attention to primary prevention in persons with symptoms of atherosclerotic disease, diabetes, and multiple risk factors, especially those associated with the metabolic syndrome (5). Therapy is targeted on lowering LDL-C values below a target value, which depends on presence of the number of other risk factors (low high density lipoprotein (HDL)-cholesterol, cigarette smoking, hypertension, family history of CAD, and male gender). For patients at the highest risk for CAD or with the highest CAD risk equivalents (the latter considered to be diabetes or a 10-year risk for CAD >20%, calculated from the Framingham risk tables), the goal is to achieve LDL-C <100 mg/dl, now considered an optimal value. For patients with two or more risk factors, the goal is to bring LDL-C to <130 mg/dl, and for those with no or one risk factor, the LDL-C goal is <160 mg/dl (5). Patients hospitalized for a major coronary event should have lipid measurements on admission or within 24h. Reliable classification of patients necessitates accuracy and standardization of LDL-C measurements.

Although the measurement of LDL-C levels is important, an easy, reliable, and suitable methodology for LDL-C has never existed in routine laboratories. β -Quantification currently is considered the reference method, but it requires ultracentrifugation, uses large volumes of serum, and is a time-consuming and expensive technique. Therefore this method is not suitable for routine laboratory testing. For that reason, most laboratories estimate LDL-C by the Friedewald Formula (FF)(6) from concentration of TC, triglycerides (TGs), and HDL-C. Friedewald formula is expressed as follows: $[LDL-C] = [TC] - ([HDL-C] + [VLDL-C])$, where the very low-density lipoprotein cholesterol (VLDL-C) concentration is estimated from the serum triglycerides concentration (in mg/dl) as $[VLDL-C] = [TGs]/5$. Although the estimation method correlates highly with β -quantification it has certain limitations: it is not valid in specimens with chylomicrons, with TGs > 400 mg/dl, or in patients with dysbetalipoproteinemia (7). Indeed, it has been recommended that the FF should be used with precaution in several pathologic states (diabetes, nephropathy, hepatopathy) even if TG concentrations are between 200 and 400 mg/dl (8). This formula (FF) assumes the ratio of total TGs to VLDL-C to be constant in all samples. However, there are some limitations for this postulation. For example, the formula will overestimate VLDL-C and underestimate LDL-C as a consequence if triglyceride-rich chylomicrons and chylomicron remnants are present in the serum specimen (hence the requirement for a fasting sample) (7). The use of the FF is also not recommended for type II diabetes, nephrotic syndrome and chronic alcoholic patients because accompanying abnormalities in lipoprotein composition render the underlying assumptions invalid for assessment of cardiovascular risk in these patients (8).

The NCEP Working Group on Lipoprotein Measurements (9) has recommended that the LDL-C concentration be determined with a total analytical error not exceeding $\pm 12\%$ ($\leq 4\%$ imprecision and $\leq 4\%$ inaccuracy) to guarantee correct patient classification into the NCEP risk categories. It is difficult to obtain this analytical quality with FF because each component's analytical error is added. These limitations create the need for alternative methods that can quantify LDL-C and can be adopted for routine use in clinical

laboratories. The aim of the present study is to assess the analytical performance of a direct immunoseparation method, and to compare it to the FF.

Materials and Methods

Samples

Blood samples were obtained from 47 patients randomly selected from an out-patient populations attending the Laboratory of Haydarpaşa Numune Hospital. Blood was collected in tubes without anticoagulant from subjects after a 12 hours fast. The samples were allowed to clot at room temperature, and serum was obtained by centrifugation at 2000g for 15 min. All direct analyses were performed in the same day.

Procedures

Direct LDL-C assay: The principle of the assay: Chylomicrons, VLD and HDL were separated by immunoseparation using separation tubes containing special latex bead. After centrifugation the cholesterol in the supernatant (LDL-C) is measured by an enzymatic – colorimetric method. The latex beads are coated with a goat antiserum, which was produced against specific human apolipoproteins (Apo A1 and Apo E). These antibodies bind chylomicrons (contain Apo A1/E), HDL-C (contains Apo A1/E), VLDL-C (contains Apo E) and IDL-C (contains Apo E). Because LDL-C doesn't contain Apo A1/E, it remains in the supernatant. The LDL-C assay (Sigma Diagnostics, USA) was performed according to manufacturer's specifications on a Hitachi 717 analyzer follows. A lyophilized calibrator provided by the manufacturer was used. LDL was isolated by the immunoseparation method according to the manufacturer's instructions: 200 μ l of LDL-C reagent was put into separations tubes and then 30 μ l of serum was added. After vortexing immediately, the tubes were incubated at room temperature for 10 min and centrifuged at 6000 rpm at room temperature for 5 minutes. The cholesterol in the filtrate was measured on a Hitachi 717 analyzer using a calibration curve suitable for low cholesterol values.

Total cholesterol, TG, and HDL-C: TC and TG levels were measured enzymatically with the CHOD-PAP (Roche Diagnostics, Germany) and lipase/GPO/PAP (Roche Diagnostics, Germany) methods, respectively, on a Hitachi 717 analyzer. The HDL-C as subsequently determined by precipitation with phosphotungstic acid and MgCl₂ (Roche Diagnostics, Germany). After incubation at room temperature for 5 minutes the apoprotein B-containing lipoproteins were sedimented by centrifugation, and the cholesterol component was measured in the supernatant with a CHOD-PAP method on a Hitachi 717 analyzer.

Friedewald Calculation: LDL-C was estimated by FF as follows: $LDL-C = TC - HDL-C - (TG/5)$.

Analytical Performance Evaluation

Precision: Two patient serum pools with medium and high LDL-C concentrations and two commercial controls (Precinorm L, and Precipath L, Roche Diagnostics, Germany) were used. Intra-assay imprecision was calculated as the mean variance obtained for 30 replicate analyses at the same time in a day. To assess interassay imprecision, aliquots of controls and pools stored at -20°C were analyzed over 10 consecutive days.

Total error: To assess the accuracy of direct assay, two control serums (Precinorm L and Precipath L, Roche Diagnostics) were used. Total error was calculated by adding the systematic error and the random error. Systematic error was calculated as follows: $\text{Systematic error, \%} = \text{mean of } [(direct\ assay - target\ value)/target\ value] \times 100$. Random error was defined as the total imprecision multiplied by 1.96 (10).

Stability study: Two serum pools were prepared and stored in aliquots at -20°C . The LDL-C concentrations were measured weekly with direct assay over 2 months.

Comparison of Methods

The LDL-C concentrations measured by the direct assay in the serum samples were compared to those calculated by FF. For this purpose samples were classified into two groups according to their TGs concentrations: (a) group 1, defined as TGs 60-308 mg/dl, (b) group 2, defined as TGs 320-695 mg/dl.

Statistical Analysis

Values were expressed as mean \pm SD. Linear regression analyses were used to assess the correlations between two methods. The t-tests were judged significant at $p<0.05$.

Results

Precision

The precision profile of the direct assay performed with the Precinorm L and Precipath L, and the patient serum pools with medium and high concentrations of LDL-C are shown in the Table 1 and Table 2. Intraassay (within run) and interassay (run-to-run) precision of direct assay was very good. The total coefficient of variances (CVs) for all four concentrations were 1.41-1.72 % for direct method. According to the NCEP performance goals, LDL-C must be measured with an imprecision $\leq 4\%$. The direct immunoseparation method has met this performance criterion.

Total Error

The total analytical error of the direct assay was $< 12\%$, as recommended by the NCEP (3) (mean 2.41%).

Sample Stability

The means of LDL-C concentrations measured by direct assay of two serum pools at baseline and after storage periods of 1-8 weeks at -20°C are presented in Fig.1. The initial concentration was considered 100%, and the plot showed no significant change in LDL-C concentrations during the 2 months.

Comparison Between Direct Assay and Friedewald Formula

The comparison of methods plot [direct assay-LDL-C (x) vs. FF (y)] in group 1 (patients with TGs 60-308 mgr/dl) showed a regression equation of $y=1.030x-0.289$ mg/dl ($r=0.9908$; $n=25$) (Fig. 2). The correlation coefficient comparing the direct assay with FF was highly significant ($P<0.0001$). The comparison of methods plot in group 2 (patients with TGs (320-716 mg/dl) showed a regression equation of $y=0.947x-24.372$ mg/dl ($r=0.7472$; $n=22$) (Fig.3). The correlation and degree of agreement between the direct assay and FF was worse in group 2 ($P<0.021$).

The relation between TGs and LDL-C levels determined by direct method and FF is shown in Fig. 4. The correlations between TGs and LDL-C concentrations measured by the direct assay and calculated with FF is concordant when TGs <400 , whereas there was a determined disagreement in the samples with TGs >400 mgr/dl.

Discussion

Many epidemiological and clinical studies have demonstrated that elevated concentration of LDL-C is a major risk factor in the development of coronary artery disease (11-14). Therefore, the Adult Treatment Panel (ATP) (3,5) focuses on LDL-C as the primary target in CAD classification and clinical management of patients at risk for CAD. Since relatively small changes in LDL-C levels can lead to change in coronary heart disease risk, it is necessary to have a reliable measurement. Many of the current techniques for determination of LDL-C in serum are cumbersome and require specialized instrumentation, which limits their use in routine practice. For that reason, routine clinical chemistry laboratories indirectly calculate LDL-C concentrations from TC, TG, and HDL-C concentrations using the FF, which assumed that the relationship between cholesterol and TGs in VLDL was constant. The FF can be performed in any laboratory but it is time consuming and combi-

Table 1. Analytical imprecision of direct assay for LDL-C, using patient serum pools

	IMPRECISION						
	Intraassay			Interassay			
	n ^b	Mean \pm SD, mg/dl	CV,%	n ^c	Mean \pm SD, mg/dl	CV,%	Total CVa, %
Medium PSP ^b	30	97.27 \pm 1.42	1.45	10	84.10 \pm 1.17	1.39	1.69
High PSP	30	263.97 \pm 2.48	0.94	10	260.20 \pm 3.81	1.46	1.55

^a Total CV, % = (CV_{intraassay} + CV_{interassay})/2
^b n, number of replicates
^c n, number of consecutive days
 CV: Coefficient of variation, LDL-C low-density lipoprotein cholesterol, PSP: patient serum pool

Table 2. Analytical imprecision and total error of direct assay for LDL-C, using control materials

	Target value mg/dl	Imprecision				Total CV, % ^a	Systematic error, % ^b	Random error, % ^c	Total error % ^d
		Intraassay		Intraassay					
		Mean \pm SD, mg/dl n ^e =30	CV, %	Mean \pm SD, mg/dl n ^f =10	CV %				
PnL	102	100.907 \pm 1.45	1.40	103.40 \pm 1.62	1.56	1.72	-1.01	3.37	2.36
PpL	292	291.14 \pm 2.98	1.02	290.08 \pm 2.85	0.98	1.41	-0.30	2.76	2.46

^a Total CV, % = (CV_{intraassay} + CV_{interassay})^{1/2}
^b Systematic error, % = mean of [(direct assay value-target value)/target value] x 100.
^c Random error, % = total imprecision x 1.96
^d Total error, % = systematic error (%) + random error(%).
^e n, number of replicate
^f n, number of consecutive days
 CV: coefficient of variation, LDL-C: low-density lipoprotein cholesterol, PnL: Precinorm L, PpL: Precipath L, SD: standard deviation

nes analytical and biological variability of three parameters and thus often fails to meet the National Cholesterol Education Program performance goals. Furthermore, it cannot be used in non-fasting samples, when TGs levels are increased and in dysbetalipoproteinemia. Therefore, the NCEP Working Group on Lipoprotein Measurement recommended the development of direct methods for LDL-C measurement (9).

We evaluated a direct immunoseparation method for LDL-C measurement that can easily be automated. Within-run and between-run precisions were always below the 2% CV, and total error was below 3%. The NCEP has clearly laid down the analytical goal for the acceptability of any new assay measuring LDL-C (3,5): the imprecision of LDL-C determinations should not exceed 4%, and the total error should be <12%. In our study, the precision of direct assay is excellent and similar to that of other published reports (15-18). Our data indicate that samples stored at -20°C for up to 2 months did not experience any important change in their LDL-C concentrations when measured by direct assay. This observation could have a practical advantage to the clinical laboratories. Recently published articles by Esteban-Salan et al (15) and Smets et al (16) confirm our findings concerning the stability of the samples.

The most commonly used method at present to estimate LDL-C is the FF. Comparison of results obtained with the direct method and LDL-C estimates based on this formula yielded an excellent correlation in the serum samples with TGs <320 mg/dl ($r=0.9908$,

$P<0.0001$), but there was a lack correlation between two methods when TGs>320 mg/dl ($r=0.7472$, $P<0.021$).

Reliable measurement of LDL-C in hypertriglyceridemic samples has always been a cause of concern (19,20). In this study, we evaluated the relation between triglycerides and LDL-C concentrations obtained with direct assay and FF. There was a good agreement between LDL-C values determined with direct assay and FF when TGs were <400 mg/dl, whereas there was a disagreement between LDL-C concentrations found by two methods when TGs exceed 400 mg/dl.

Smets et al (16) have reported that there was a significant correlation between direct method and FF, but FF was unsuitable when TGs exceed 200 mg/dl. Nauck et al (21) have reported that homogeneous methods for LDL-C do appear to be significantly less susceptible to interference from increased TGs than the Friedewald calculation. Scharnagl et al (22) have reported that the Friedewald calculation was invalid for the determination of LDL-C in samples in which low concentrations of LDL-C have been achieved by LDL apheresis, and this finding might also be of relevance to the monitoring of patients being treated with lipid lowering drugs.

In conclusion, the direct immunoseparation method for determination of LDL-C provides an improvement over the currently used FF: (1) it is easily automated and rapid, (2) both imprecision and bias meet the NCEP performance goals, (3) it has good analy-

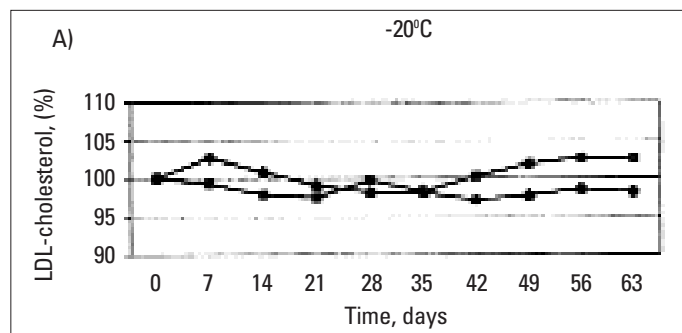


Figure 1. Effect of storage at -20°C on the measurement of LDL-C by direct assay.

The values represent the percentages of recovery with respect to values obtained in fresh serum pools.

LDL-C: low density lipoprotein cholesterol

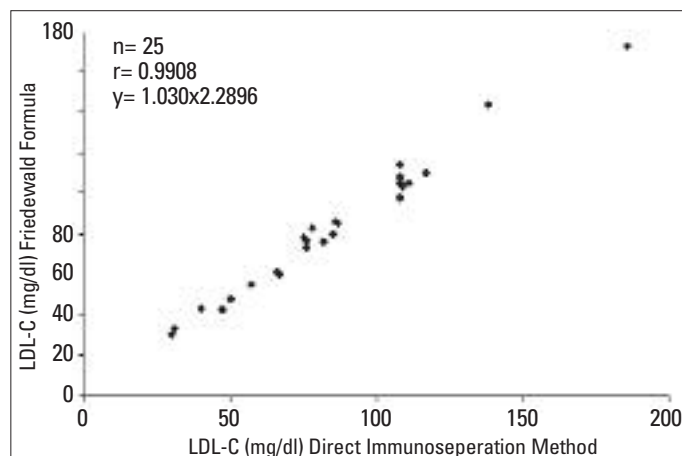


Figure 2. The correlation of LDL-C levels obtained by direct immunoseparation method with LDL-C levels calculated with FF in group 1 (patients with TGs 60 - 308 mg/dl)

FF: Friedewald formula, LDL-C: low-density lipoprotein cholesterol, TGs: triglycerides

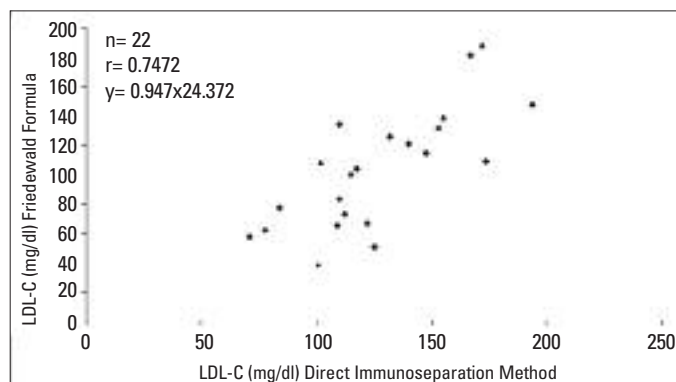


Figure 3. The correlation of LDL-C levels obtained by direct immunoseparation method with LDL-C levels calculated with FF in group 2 (patients with TGs 320-716 mg/dl);

FF: Friedewald formula, LDL-C: low-density lipoprotein cholesterol, TGs: triglycerides

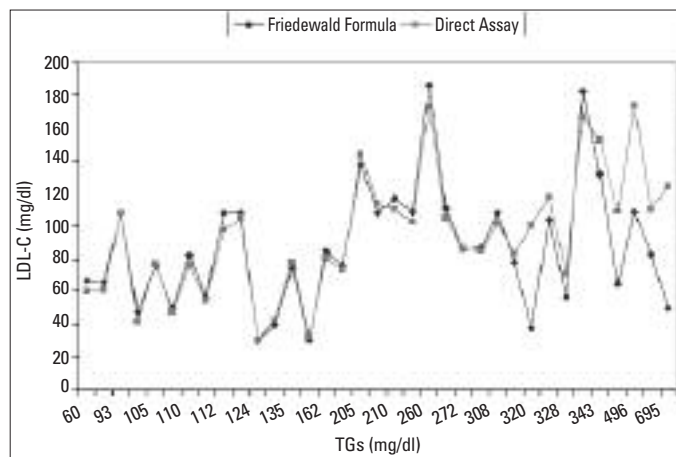


Figure 4. The correlations between TGs and LDL-C concentrations measured by the direct assay and calculated with Friedewald formula in two groups.

LDL-C: low-density lipoprotein cholesterol, TGs: triglycerides

tical performance characteristics, (4) it gives reliable results with hypertriglyceridemia, (5) LDL-C is measured directly and not estimated from other parameters, thereby reducing analytical and biological variability.

Basic Clinical Interpretation

The direct assay is a precise and acceptably accurate method. It represents an improvement in the measurement of LDL-C concentration in samples with increased TGs or samples collected postprandially and may assist in the identification of individuals at increased risk of CAD and the management of patients with hyperlipoproteinemia.

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