EFFECT OF BUFFERS USED IN HOMOGENOUS ASSAY FOR THE MEASUREMENT OF HDL- CHOLESTEROL IN SERUM

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SERUM HOMOJENOZ HDL KOLESTEROL ÖLÇÜMÜNE TAMPONLARIN ETKİSİ

Özet: *Sunulan çalışmanın amacı serum homojenoz HDL-kolesterol ölçümlerinde kullanılan Polianyon Polimer (PPD) metodu için iki farklı tamponun 1998 yılında NCEP tarafından bildirilen performans kriterlerine uyumluluklarını incelemektir. Birinci PPD metodunda (PPD-1) Ftalik Asit tamponu (60 mmol/L, pH= 5.7) kullanılmakta olup ikincisinde ise (PPD-2) MES tamponu (2n-morpholine ethanosulphonic acid, 30 mmol/L, pH= 6.3) kullanıldı. Bu metodlar immuninhibisyon metoduyla (II) birlikte Technicon RA-XT analizörüne uygulandı. II metodu beta lipoproteinlere karşı oluşturulmuş antikorların tüm HDL dışı lipoproteinleri bloke etme prensibine dayanmaktadır. Bu üç metodun performansı ultrasantrifüj metoduyla kıyaslandı.*

Araştırmada kullanılan 96 numuneden 22 tanesinde trigliserid yüksek (> 200 mg/dl) , 28 tanesinde bilirubin yüksek (> 1.5 mg/dl) ve 46 tanesinde de hem bilirubin hem de trigliserid değerleri yüksek idi. Precision çalışmasında; with-in run ve between-run değerleri sırasıyla II için % 4.21, 2.38; PPD-1 için %2.07, 20.13 ve PPD-2 için % 2.10, 4.27 idi. Sistematik Hata değerleri II için % 5.21, PPD-1 için 13.2 ve PPD-2 için 22.34 idi. Random Hata değerleri II için % 4.66, PPD-1 için 39.4, ve PPD-2 için 8.37 idi. Total Eror değerleri II için %9.87, PPD-1 için 52.66 ve PPD-2 için 30.7 idi. Uyum sınırları istatistiği uygulandığında ise tüm homojenoz metodlar arasında PPD-1 en fazla variabilite gösteren metod olarak bulundu. Sonuç olarak HDL-C ölçümü için ftalik asid tamponu kullanılan PPD-1 metodu 1998 NCEP kriterlerine uyum göstermemektedir

Anahtar Kelimeler: *HDL-C, Direct HDL-C, Homojenoz HDL-C.*

Summary: *The purpose of this study was to compare three kinds of homogenous HDL-C methods and also evaluate two different buffers used in polyanion polymer and detergent (PPD) homogenous assay for the measurement of HDL-Cholesterol (HDL-C) in serum for their ability to achive the performance goals established by NCEP.*

The first PPD assay (PPD-1) contained phthalic acid buffer (60 mmol, pH 5.7), and the second (PPD-2) contained MES buffer (2n- morpholine ethanosulphanic, 30 mmol, pH: 6.3). Both of two PPD assays were conducted on RA-XT Technicon analyzer together with the immunoinhibition (II) method.

A comparison study was performed, with 96 samples (22 sera with high triglyceride (TG), 28 sera with high bilirubin (Bil), and 46 both high triglyceride and bilirubin levels) Performances of the direct methods were compared to standart ultracentrifugal (UC) method for HDL-C assay.

Precision data of with-in run and between-run were: 4.21 and 2.38% for II , 2.07 and 20.13 % for PPD-1 : and 2.10 and 4.27 % for PPD-2 methods respectively.

Systematic Error results of II , PPD-1 and PPD-2 were; % 5.21, % 13.2 and 22.34 and Random Error results were % 4.66, % 39.4 and % 8.37 finally, results of Total Error were % 9.87, % 52,66 and % 30.7 respectively.

Regarding the agreement limits for intermethod comparison, PPD-1 assay had the worst results with the minimum limits of -34.229. The results of II and PPD-2 assays were found to be similar and superior to that of PPD-1

In conclusion, on comparison of methods, PPD-1 method for HDL-C assay which contained Phthalic acid buffer showed no clinically acceptable performance and buffer systems have to be taken into consideration in order to approach NCEP goal in HDL-C assays

Key Words: *HDL-C, Direct HDL-C, Homojenoz HDL-C*

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Türk Biyokimya Dergisi
2000, Cilt: 25, Sayı: 4

INTRODUCTION

Studies indicate that HDL-C is an independent factor for the prediction of Coronary Heart Disease (CHD) (1-2) Accordingly, low HDL-C <35 mg/dl (0.91 mmol/L) is considered as equivalent to nonlipid risk factors such as smoking or hypertension and high HDL-C >60 mg/dl (1.55 mmol/L) is a negative risk factor, thereby compensating for the presence of a positive risk factor (3,4).

In most clinical chemistry laboratories, determination of HDL-C is being performed manually in a two-step procedure. First, apolipoprotein (apo) B-containing (nonHDL) particles are precipitated with solutions containing divalent cations (magnesium, calcium, or manganese) and polyanions, e.g. sulfated polysaccharides (heparin or dextran sulfate), sodium phosphotungstate; then, the precipitated lipoproteins are removed by centrifugation, and HDL-C is determined by measuring the cholesterol remaining in the supernatant liquid. In the presence of high triglycerides, precipitation is often incomplete, requiring manual dilution, repeated precipitation and reanalysis (5)

Traditional methods cause waste of a great deal of man power and time in laboratories which have a high potential of patients. The requirements for manual handling and special treatment make it an expensive test, compared with other lipid measures. Second generation procedures still require sample precipitation.

In recent years, to overcome the problems encountered in traditional testing for HDL-C, several new, direct methods have been developed which have certain differences from each other. The third generation procedures are the direct or homogeneous HDL-C assays, which use only a small sample volume and do not require the centrifugation and precipitation steps. These methods eliminate the need for pretreatment steps since the reaction of cholesterol in the non-HDL lipoproteins is selectively inhibited.

One direct HDL method, for example, uses α-cyclodextrin sulfate as sequestering agent and polyethylene glycol (PEG)-modified cholesterol esterase to restrict the reaction to the cholesterol contained in HDL particles (6). Another method combines the use of PEG 4000 to sequester VLDL and LDL and antibodies to apos B and C-III to produce aggregates of the chylomicrons, VLDL and LDL that will not react with cholesterol esterase (7). Additional methods include direct inhibition of the reaction of non-HDL cholesterol by use of antibodies to β- lipoproteins (8) or the use of polyanions and polymers to complex apo-B containing lipoproteins, which are stabilized in an unreactive form with a detergent that solubilizes HDL (9). In direct (homogenous) reaction systems, an inhibitor is mixed with the serum and, after incubation for complexation of β- lipoproteins, enzymatic reagents for the determination of cholesterol are mixed directly with the pretreated serum sample. The common purpose of all direct methods is assay of HDL-C in a single cuvette without manual precipitation and application of this process to automation. An additional advantage of most direct HDL-C methods is that, it is not affected from high triglyceride concentrations. Recently, investigations have shown that triglycerides as high as 1800 mg/dl have minimal effect in two different direct methods (6,10). Similarly, it has been pointed out that increased bilirubin levels may interfere with HDL-C assays due to reductive effects (12,22).

Inaccuracy and imprecision goals of NCEP (National Cholesterol Education Program) required total error of < 13%. However, for the standardization, CDC's Cholesterol Reference Method Laboratory Network (CRMLN) purposes to meet separate accuracy and precision goals (14). The 1998 performance goals recommended by the NCEP that are consistent with the total error goal are a bias

vs the accuracy base < 5% and a precision of CV< 4% at HDL-C > 1.09 mmol/L (42 mg/ dl) and SD < 0.044 mmol/L (1.7 mg/dl) at HDL-C< 1.09 mmol/L (42 mg/dl).

In this study, we have performed an investigation for the comparison of two homogenous methods namely Immuninhibition (II) and synthetic polymers and polyanions (PPD) methods of HDL-C determination with the reference ultracentrifuge method. Since PPD methods we used were based on two different buffers, we numbered them as PPD1 and PPD2. To evaluate whether these methods are affected from high triglyceride and high bilirubin levels that are known to be very common for HDL-C assays, all the comparison studies were made in these type of sera. We also compared the results of the study with the NCEP goals for accuracy and precision. All data presented here about the comparison of direct methods, aimed to be beneficial to the workers of other clinical chemistry laboratories.

MATERIALS AND METHODS

Samples and controls

We collected 96 blood samples for HDL-C assays at İbn-i Sina Hospital. These 96 samples were composed of 22 samples with high triglyceride, 28 samples with high bilirubin and 46 samples with both high triglyceride and bilirubin levels. Ten normal controls with a mean HDL-C concentration of 1.15 mmol/L (0.78 - 1.53 mmol/L) (44.6 mg/ dl) were pooled, diluted by two and included in the study. These samples were allowed to clot at room temperature for 1 h, and then the serum samples were obtained by centrifugation at 1500 g for 10 min. at room temperature. All assays were performed within 2 days of blood collection. Meanwhile samples were kept at +4 °C.

Homogenous HDL-C Assays

PPD-1 Method: Reagent supplier is Biocon (Germany). The principle of the PPD-1 depends on synthetic polymers and polyanions contained in the first reagent adsorbed on the surface of LDL, VLDL and chylomicrons and transform these lipoproteins to a stable form. By the second reagent HDL particles are solubilized. Only the HDL-Cholesterol is subject to color reaction which is catalysed by cholesterol esterase and cholesterol oxidase. H_2O_2 in the first reaction, react with phenol and 4-aminoantipyrine which catalysed by peroxidase. The intensity of coloured product quinoneimine dye is proportional with the HDL-C in the sample. Constituents of reagent 2 were, Phthalic acid buffer pH 5.7 (60mM), PEG mod. Cholesterol oxidase (>1 kU/L), PEG mod. Cholesterol esterase (>5 kU/L), Peroxidase (30 kU/L), N,N-BSBmT (0.4 mmol/L) .

PPD-2 Method: The reactives were supplied from bioMérieux (France). The principle of this method was similar to PPD-1. Synthetic polyanions present in Reagent 1 are adsorbed on LDL and VLDL and transform these proteins into a stable form (phase 1).

Free HDL particles are solubilized by the detergents present in Reagent 2, enabling the cholesterol originating from the HDL fraction to be assayed using a standard enzymatic method in the presence of cholesterol oxidase and cholesterol esterase (phase 2). Reagent 1 contents were polyanion, 4-amino-antipyrine (0.67 mM), MES buffer (2 (n-morpholine) ethanosulphonic acid monohidrate, pH = 6.3), and preservative. Reagent 2 contents were Cholesterol oxidase (1.6 IU/ml), Cholesterol esterase (1.25 IU/ ml), Peroxidase, detergent, and sulfobutyrl toluidine (DSBmT) (1 mmol/L).

Immunoinhibition method: Direct HDL-C reactive which was based on the Immunoinhibition principle (supplied from Sigma ,USA) and composed of the following reactives: Anti human β lipoprotein antibody in reagent-1 binds to lipoproteins (LDL, VLDL and chylomicrons) other than HDL. The antigen-antibody complexes formed block enzyme reactions when reagent-2 is added. Cholesterol

esterase and cholesterol oxidase in reagent-2 react only with HDL-C.

Modified Ultracentrifuge Method for HDL-C : A combined ultracentrifugation-precipitation assay was used as the comparison method of reference. In this method, 230 µL serum samples were centrifuged at 300 000g for 3 h at 4 °C in a TL 100 rotor with 7x20 mm polyallomer tubes (Beckman- Coulter, Optima TL100, Palo Alto, CA). The tubes were then sliced to isolate >1.006 kg/L infranatant to discard the fraction containing the VLDL and chylomicron particles. The volume of the infranatant was restored to the original sample by isotonic saline (9 g/L), and HDL-C was quantified after $MgCl_2$ (50 mmol/L) and dextran sulfate (Mr 50 000; concentrations in the specimen 1 g/L) precipitation of the particles containing LDL, intermediate-density lipoproteins, and lipoprotein (a) (11).

All of the comparison studies of direct HDL-C were performed in RA-XT (Technicon) analyser. Among the serum samples we studied, triglyceride levels higher than 6.78 mmol/L (600 mg/dl) were selected as hypertriglyceridemic, and bilirubin levels higher than 136.8 µmol/ L(8 mg/dl) were selected as hyperbilirubinemic for interference studies.

Statistical Methods:

Precision Study: To perform within-run and between-run precision, we used two sets of pooled human sera. The first was prepared by mixing 10 normal sera with mean HDL-C concentration of 1.15 mmol/L (control I) and the second pool was prepared by ½ dilution of the first serum pool with isotonic saline making up a mean value of 0.59 mmol/L (control II) . Both of the controls were evaluated 10 times by three different reactives in the same day for with in- run precision and 3 times during the sequental 7 days for between- run precision. All of them were stored in aliquots at –20°C until analysis. Standart deviations and coefficients of variations were calculated. The mean values of Total Cholesterol, Triglyceride and Bilirubin in the control I were 5.48 mmol/L(211.6 mg/dl), 1.71mmol/L (151.3 mg/dl) and 17.61 µmol/L (1.03 mg/dl) respectively.

Total error: Total error is the sum of systematic error plus random error (21). Systematic error was calculated from the linear regression equation y =bx + a, where b is the slope of the regression equation, and a is the y-axis intercept. Systematic error at an HDL-C concentration of xc is defined as the absolute value of yc-xc, where yc=bxc + a. Random error is 1.96 x the SD of the run to run precision study.

Intermethod Comparison: To compare the HDL-C results of each three homogenous method with the result of UC method, and for the investigation of possible interferences depends on the increase of triglyceride and bilirubin levels, Passing –Bablok Regression (16) and "Agreement limits" formulation described by Altman and Bland (15), was used.

RESULTS

A total of 96 sera were analysed with each of the homogenous methods for intermethod comparisons. These samples were composed of 22 sera with high triglyceride, 28 sera with high bilirubin and 46 sera with both high triglyceride and bilirubin levels.

The mean ± SD values of Total Cholesterol (mmol/L), Triglyceride (mmol/L) and Bilirubin (µmol/L) in TG high sera (n= 22) 6.32 ± 2.40, 8.22 ± 0.29, 16.8 ± 35.9; in Bilirubin high sera (n= 28) 4.24 ± 2.38, 1.60 ± 0.11, 273.8 ± 44.4; in both TG and Bilirubin high sera (n= 46) 4.92 ± 1.44, 7.52 ± 0.91, 172.6 ± 63.7 respectively.

Two human pool sera were used to assess the precision of the HDL-C assays (Table-I). The total CVs ranged from 4.84 to 5.27 for II assay, 20.24 to 19.61 for PPD-1 assay and 4.76 to 6.69 for PPD-2 assay, respectively. Therefore, only II assay met the performance criteria for HDL-C imprecision of < 4% established by both the CDC and NCEP (14)

According to the percentage error results of the three methods, the most convenient test seemed to be the II method with total error results of 9.87 for control-1 and 17.61 for control-2 as shown in Table-II. As both the systematic and random errors contributed to the calculation of total error, lowest total error was calculated for II assay. The highest total errors were found for the PPD-1 assay.

Intermethod comparisons were made by two ways. The first method was the Passing- Bablock regression analysis and the second was the calculation of agreement limits.

When we estimated the parameters of the regression lines according to Passing and Bablock, in the high triglyceride group, the slopes relating all three homogeneous assays to the UC assay were not significantly different from 1.00 and, the intercepts belonging to PPD-1 and PPD-2 produced significantly different HDL-C values from the reference UC procedure. In high bilirubin sera, only the slope for PPD-1 was significantly different from 1.00 and intercepts of all three methods were found to be significantly different from 0.00. In the high TG and bilirubin sera, the slopes for all three methods were nearly same and not significantly different from 1.00 .The intercepts for II and PPD-2 methods were significantly different from 0.00. As a result, the best correlation between three methods and reference UC method were detected in II method for all groups. The correlations of II method to the reference UC method in these groups were r= 0.726, 0.816, and 0.955 respectively (Table-III).

The value of agreement limits which was the other way of intermethod comparison, showed that the differences between II and PPD-2 assays in hypertriglyceridemic samples were not high. However, PPD-1 assay had the worst results with the minimum limits of -34.229. Additionaly, there was no difference detected between the agreement limits of three methods in hyperbilirubinemic samples.

Table-I Analytic Precision of three homogenous HDL-C methods

Methods	With-in run precision			Between-run precision			Total	
Control-1	Mean	SD	CV%	Mean	SD	CV%	SD	CV%
II	1.19	1.95	4.21	1.20	1.11	2.38	2.24	4.84
PPD1	1.49	1.2	2.07	1.23	9.62	20.13	9.69	20.24
PPD2	1.01	0.82	2.10	0.95	1.58	4.27	1.78	4.76
Control-2								
II	0.60	0.63	2.72	0.62	1.09	4.51	1.26	5.27
PPD1	0.66	0.92	3.57	0.53	3.95	19.28	4.06	19.61
PPD2	0.52	0.95	4.68	0.49	0.92	4.78	1.32	6.69

HDL-Cholesterol= mmol/L

Table -II: Percentage Error results of three homogenous HDL-C methods

Methods	Systematic Error %		Random Error %		Total Error %	
	Control-I	Control-II	Control-I	Control-II	Control-I	Control-II
II	5.21	8.77	4.66	8.84	9.87	17.61
PP - 1	13.2	7.93	39.46	37.78	52.66	45.71
PPD-2	22.34	15.28	8.37	9.36	30.71	24.64

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Table-III : Passing- Bablok Regressions

TG HIGH	Slope	Intercept	r
II/ UC	1.45	-13.19	0.726
PPD-1/ UC	2.0	-44.50**	0.214
PPD-2/ UC	0.667	11.33**	0.599
BİL HIGH	**Slope**	**Intercept**	**r**
II/ UC	0.97	8.162**	0.816
PPD-1/ UC	0.664*	10.769**	0.751
PPD-2 / UC	0.876	5.109**	0.732
TG AND BIL HIGH	**Slope**	**Intercept**	**r**
II/ UC	1.07	4.85**	0.955
PPD-1/ UC	0.87	3.74	0.719
PPD-2/ UC	0.91	7.708**	0.876

- * Slopes are significantly different from 1.00
- ** Intercepts are significantly different from 0.00
- Concentrations are given in mg/dl

Table –IV: Agreement limits of three methods in TG high, Bilirubin high and both TG and Bilirubin high sera

Methods	Agreement Limits	
	Minimum	Maximum
TG HIGH		
II	-12.359	18.930
PPD-1	-34.229	13.829
PPD-2	-11.569	14.51
BILIRUBIN HIGH		
II	-11.17	23.708
PPD-1	-17.819	20.379
PPD-2	-16.57	17.401
TG AND BILIRUBIN HIGH		
II	-4.092	17.162
PPD-1	-20.69	23.34
PPD-2	-5.17	13.80

The results of II and PPD-2 assays were found to be similar and superior to that of PPD-1 whereas, the PPD-1 assay results were different from the others in both high TG and bilirubin sera .

DISCUSSION

In this study we evaluated the performance of three homogenous HDL-C assays that can be readily adapted to automated analyzers and compared the results with those obtained by the reference UC method. The direct homogenous methods are principally different assays as the first method (PPD) obtained from two different manufacturers, is described as PPD- 1and PPD-2. In this method; synthetic polymers, polyanions, and detergents coupled with a conventional enzymatic cholesterol assay is employed for the quantification of HDL-C. The second method (II) utilizes antibodies for the separation of non-HDL lipoproteins coupled to a conventional enzymatic cholesterol assay.

Commonly, the measurements of two analytical methods are compared by a regression analysis procedure, which allows the detection of a possible constant systematic difference (intercept deviation from zero) and a proportional systematic difference (slope deviation from unity). In recent years, statistics in the form of a paired t- test is not found to be appropriate for the analysis of method comparison data because it might be misleading in the presence of a systematic proportional difference. Thus, we used more valid methods such as Passing and Bablok, and Bland and Altman. The latter has gained increasing popularity as a tool for evaluation of method comparison.

Accurate and precise HDL-C measurements are essential for effective application of

The National Cholesterol Education Program (NCEP) treatment guidelines. Recently, The Cholesterol Reference Method Laboratory Network (CRMLN) developed, evaluated and standardized a comparison method (DCM) to overcome the impracticabilities of the expensive and labor-intensive reference method for HDL-C. This method has several practical advantages over the use of the reference method for HDL-C (23).

NCEP goals for HDL-C 1998, demand a total error of <13% (14). In the current study, PPD-1 and PPD-2 assays have not met these criteria . In PPD-2

assay, this result may have arised from the high systematic error caused by an incorrect calibration (positive y-intercepts in all high TG, high bilirubin and both high TG and bilirubin samples in the intermethod comparison). But, the same interpretation can not be made for the PPD-1 assay because of the different intercepts in all three samples. The II assay had the lowest total error.

Standardization of HDL-cholesterol is needed for risk assessment. Indeed, the biases on the HDL-Cholesterol values found by various precipitation methods were highly variable. Cobbaert et al assessed the total error (TE) of currently used routine HDL-C in native human sera by 25 clinical laboratories. Of the homogenous HDL-C method group, the range of interlaboratory biases were the largest in the polymer/ polyanion assay group, and the maximum TE (around 15%) was largest for the same method group (23). They explained these results with the improper reagent formulation and/ or application rather than improper value assignment of the calibrator. This weak performance might arise from the inconvenient buffer system as observed in our results.

The results of this study demonstrate that , II assay has the best analytical precision of the all HDL-C methods we assessed. In this study, we used different kind of buffers which are widely found in commercially available PPD reagents. In one PPD assay (PPD-1), we used phthalic acid buffer (60 mmol, pH 5.7), and in the other (PPD-2) MES buffer (2n- morpholine ethanosulphanic, 30 mmol, pH: 6.3). The weak correlation between PPD-1 and the UC method, may be attributed to unsuitable buffer environment of PPD-1 reactive, whereas the PPD-2 based on the same principle is more precise than PPD-1.

It has been observed by Kondo et al. in a study on lipoprotein visualization by electron microscopy that polyanion-polymer found in the first reaction of homogenous HDL-C assay. They confirmed that polyanion and synthetic polymer in reagent 1 aggregated with nonHDL lipoproteins and only the HDL in these aggregates selectively resolved in the second reaction, as indicated in the specifications of the manufacturer. In detail, the detergent in reagent 2 selectively resolved HDL on the surface of the aggregates, in which HDL was denuded from the inside. After separation of HDL was carried out by ultracentrifugation, fractions including HDL were dialyzed against phosphate buffered saline (25 mmol/L phosphate buffer ,pH = 7.4) this pH value is closer to that of our PPD-2 method (26). Thus, this study confirms our result about the buffer effect on activity of HDL-C. On the other hand, this difference in activity of PPD-1 and PPD-2 method for HDL-C assay, may arise from the different pKa values of these buffers used in these PPD methods.

When we evaluated the results with respect to the agreement limits, II and PPD-2 assays showed better agreement with the UC procedure. Even in high TG and bilirubin samples, very close agreement limits were determined by these two assays.

A common observation of the present study and many other reports is that homogenous assays tend to overestimate HDL-C in a range of 10%. HDL is composed of particles that markedly differe by physicochemical characteristics and apolipoprotein composition (17-19). Some particles are together with apo-E and the others without apo-E. It has been suggested by some investigators that apo-E containing HDL particles are not included in some of the HDL-C assays based on precipitation by several methods (20). This feature is known to occur especially in hypertrigliceridemic sera. In the present study, hyperlipidemic sera might have interfered with the reactives present in PPD-1 assay. In sera with high trigliceride level, the common problem is the weakness of the reactives to precipitate VLDLs resulting in the positive bias on the HDL-C results.

As a conclusion, PPD-1 method for HDL-C assay does not display clinically acceptable performance

upon method comparison and buffer systems and their pKa values have to be taken into consideration to approach 1998 NCEP goal in HDL-C assay.

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