Research Article

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Comparison of the modified polyacrylamide gradient gel electrophoresis and high-performance liquid chromatography methods in determining LDL size

LDL boyutunun belirlenmesinde modifiye poliakrilamid gradyent jel elektroforezi ile yüksek-performanslı sıvı kromatografisi metotlarının karşılaştırılması

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Abstract

Objectives: In this study, we aimed to compare modified Krauss polyacrylamide gradient gel electrophoresis (PAGGE) and high-performance liquid chromatography (HPLC) methods in classification, quantification, and separation of lipoproteins and determining low-density lipoprotein (LDL) size.

Methods: Blood specimens were obtained from eighty-seven volunteers. We measured LDL size using the PAGGE method and HPLC method with total cholesterol (TC) and triglyceride (TG) peaks. In the PAGGE method, Coomassie Brilliant Blue (CBB) staining was used instead of Sudan black staining, unlike the original method. The relationship between PAGGE and HPLC methods was evaluated by Pearson correlation test and Passing-Bablok regression analysis. Agreement between them was evaluated by Kappa analysis and Bland-Altman plots.

Results: Statistically significant correlation was found between the LDL size with PAGGE and HPLC methods under the cholesterol curve (HPLC-TC) ($r=0.924$, $p<0.001$). Similarly, there was a statistically significant correlation between PAGGE and HPLC methods under the TG curve (HPLC-TG) ($r=0.910$, $p<0.001$). In the PAGGE method, within-day precision was found as 2% and between-day precision as 3%. It was determined agreement between HPLC-TC vs. HPLC-TG methods and HPLC-TG vs. PAGGE methods was higher than HPLC-TC vs. PAGGE (Kappa values; 0.68, 0.71, and 0.44, respectively).

Conclusions: The PAGGE method can be a reliable method for measuring LDL size. HPLC method under cholesterol and triglyceride peaks may be used in clinical practice interchangeably, but clinical decision limits should be different. In addition, our study demonstrated that measurement methods for LDL size could be simplified with several modifications.

Keywords: atherosclerosis; coronary artery disease; high-performance liquid chromatography; LDL size; polyacrylamide gradient gel electrophoresis.

Öz

Amaç: Bu çalışmada, düşük dansiteli lipoproteinin (LDL) boyutunun belirlenmesi, sınıflandırılması ve ayrılmasında Modifiye-Krauss poliakrilamid jel elektroforezi (PAGGE) ve

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yüksek-performanslı sıvı kromatografisi (HPLC) metotlarının karşılaştırılması amaçlanmıştır.

Gereç ve Yöntem: Seksen-yedi sağlıklı bireyden kan örneği alınmıştır. Hem Total Kolesterol (TC) hem de Trigliserid (TG)'e göre etkileşimli LDL-boyut ölçümü PAGGE ve HPLC metotları gerçekleştirilmiştir. PAGGE ve HPLC metotları arasındaki ilişki Pearson korelasyon testi ve Passing-Bablok regresyon analizi kullanılarak değerlendirilmiştir. Uyum Kappa analizi ve Bland-Altman eğrileri kullanarak değerlendirilmiştir.

Bulgular: Kolesterol eğrisi altında LDL-boyutunun belirlenmesinde PAGGE ve HPLC (HPLC-TC) metotları arasında istatistiksel olarak anlamlı korelasyon saptanmıştır. ($r=0.924$, $p<0.001$). Benzer olarak, TG eğrisi altında da LDL-boyutunun belirlenmesinde PAGGE ve HPLC (HPLC-TG) metotları arasında istatistiksel olarak anlamlı korelasyon saptanmıştır ($r=0.910$, $p<0.001$). PAGGE metotunda gün içi presizyon %2 olarak saptanmışken, günler arası presizyon %3 olarak belirlenmiştir. HPLC-TC ve HPLC-TG arası uyum ile HPLC-TG ve PAGGE arasındaki uyumun HPLC-TC ve PAGGE arasındaki uyuma göre daha yüksek olduğu saptanmıştır. (Kappa değerleri; 0.68, 0.71, ve 0.44, sırasıyla).

Sonuç: PAGGE metotu, LDL boyutu ölçümü için güvenilebilir bir metottur. Kolesterol ve trigliserit'e göre etkileşimli LDL boyut ölçümü açısından HPLC metotu, klinik uygulamada birbirlerinin yerine kullanılabilir olsa da, klinik karar limitleri farklı olmalıdır. Ek olarak, çalışmamızda LDL ölçüm metotlarının farklı düzenlemeler ile basitleştirilebildiği de gösterilmiştir.

Anahtar Kelimeler: aterosklerozis; koroner arter hastalığı; LDL boyutu; poliakrilamid gradyent jel elektroforezi; yüksek-performanslı sıvı kromatografisi.

Introduction

Atherosclerosis is a progressive disease of the arterial vasculature resulting in the development of coronary heart disease and stroke that are the leading causes of death and significant socioeconomic burden in both developed and developing countries [1]. The most commonly recognised risk factors of atherosclerosis include male gender, family history, hypertension, diabetes mellitus, and hypercholesterolemia [2]. In addition, atherosclerosis is closely associated with age, smoking, alcohol abuse, and a sedentary lifestyle [3]. In the last two decades, both preclinical and clinical studies have revealed that disrupted lipid profile integrated with inflammation plays a critical role in the development of atherosclerosis [4].

Among lipid profile variables, the elevated plasma concentration of low-density lipoprotein (LDL) is a well-established risk factor for atherosclerosis [5]. LDL is a lipoprotein involved in the transport of cholesterol in the bloodstream and differs from other lipoproteins with its specific apolipoprotein, size, and function. LDL cholesterol level is among the crucial cardiovascular risk factors and is used as a treatment and follow-up target criterion in many lipids lowering treatments [6].

Today, the development of atherosclerosis despite the absence of numerous risk factors has prompted researchers to determine new risk factors for the prediction and prevention of the disease. Despite the controversy about the clinical utility of various risk markers of cardiovascular disease, there is a universal agreement that LDL plays a critical role in the pathogenesis of atherosclerosis [7]. The size and composition of LDL have been associated with the development of atherosclerosis [8]. Studies have shown that reduced LDL size is a significant predictor of coronary disease [9]. Small-sized LDL particles can cross the endothelial barrier, enter the subendothelial space of the arterial wall, and contribute to the development of atherosclerotic diseases [10].

The complex biochemical structures of lipid contents make the measurement of LDL size challenging. Currently, there is no standardised method for LDL size measurement yet [11]. The most commonly used methods for determining LDL size include ultracentrifugation, polyacrylamide gradient gel electrophoresis (PAGGE), high-performance liquid chromatography (HPLC), and nuclear magnetic resonance (NMR) spectroscopy [12]. Ultracentrifugation is considered a traditional method used to determine lipoprotein classes and to separate lipoproteins from plasma or serum samples [13]. However, this technique is time-demanding and requires large volume samples and great attention during the separation process [14]. PAGGE method, which allows the measurement of LDL actual size is simpler and less expensive. However, measurement of small dense LDL is confined to subjects with a normal lipid profile but a positive family history of coronary artery disease [12]. HPLC is an alternative method for the classification and quantification of lipoproteins based on the differences between particle sizes, and it has also been used as an alternative method to ultracentrifugation [15] and NMR spectroscopy, which is a relatively newer technique [16]. There is no general consensus on which of these methods alone or in combination with the others is superior, and each of these techniques has its own pros and cons. Therefore, studies aiming to develop reliable, simple, inexpensive, and easily accessible alternatives to measure LDL size continues. In this study, we aimed to compare the

PAGGE and HPLC methods in the classification, quantification, and separation of lipoproteins and in determining LDL size.

Materials and methods

Patients

The study was carried out in collaboration with the Gulhane Military Academy Hospital Laboratory (Ankara, Turkey) and Keio University Laboratories (Tokyo, Japan). The procedure was approved by the Gulhane Military Medical Committee Academic Board (dated September 26, 2005, decision no: 34) following the ethical standards established by the institution, and all procedures were applied in accordance with the Helsinki Declaration. Informed consent was obtained from all individual participants included in the study.

Eighty-seven volunteers were included in the study. Blood specimens were taken into a BD SST II serum separator tube after at least 8 h of fasting and centrifuged at 1,200 g for 10 min. The serum samples were transferred to secondary tubes and stored at −80 °C. Before the measurements, all samples were thawed once, and LDL sizes were analysed simultaneously by both PAGGE and HPLC methods on the same day.

Lipid analysis

Triglyceride, total cholesterol, HDL cholesterol, and LDL cholesterol (triglyceride>400 mg/dL) concentrations were analysed by enzymatic methods in the Olympus AU 2700 (Olympus Co. Ltd., Tokyo, Japan). LDL-C was calculated by Friedwald formula for patients with triglyceride concentrations up to 400 mg/dL.

Sample preparation

A 10 µL serum sample, 40 µL sample preparation solution (31% sucrose, 0.06% Na-EDTA, 0.01% bromophenol blue) and as internal standard 10 µL of apoferritin (5.1%, Merck&Co) were mixed.

Standards

We used standard LDL particles obtained with the ultracentrifugation technique (Sysmex 3400, Eurekos, Copenhagen, Denmark) and measured its size by negative tungsten staining under electron microscopy as 26.36 nm [17]. Also, the high-molecular-weight (HMW) protein standards kit (Pharmacia, Pharmaceutical company, New Jersey, USA) includes thyroglobulin (17.0 nm) and apoferritin (12.2 nm) was also used as standards. We utilised these three substances of known diameter to plot a linear calibration curve and determine LDL size (Supplemental Figure 1).

PAGGE method

The modified Krauss method was developed by Tsukamoto et al. in 2004 by simplifying the original PAGGE method described for the first time by Krauss et al. in 1982 [18, 19]. Changes in the modified method include the use of internal standard and high-molecular weight agents and protein staining instead of lipid staining.

In this modified PAGGE method, we had to use PAGGE (DAIICHI Chemical Company Cassette Electrophoresis Unit "DPC" Model DPE-2210 of LDL on 20–150 g/L polyacrylamide gradient gels [MULTI GEL 2/15, gel dimensions 84 mm (wide), 90 mm (height) and 1.0 mm (thickness)] Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan) to prevent free fatty acids from binding to lipoproteins which cause to increases the negative charge and affects lipoprotein migration.

Before applying samples, gels were received pretreatment with 20 V for 15 min. After adding 5 µL samples to each well, two-stage electrophoresis was applied under tris-borate buffer, pH 8.3; first at 75 V for 15 min and second at 125 V for 24 h. At the end of this process, the gel was freed from the plate and stained in the 0.4% CBB/R250 solution for 4 h. And then the gel was decolourized four times using acetic acid methanol solution (Dai-ichi Karkaria, Churchgate, Mumbai, India).

The gel image was transferred to the computer system using a scanner (EPSON GT-7600, Epson Scanners, Epson America Inc., Los Alamitos, CA, USA) and was measured using a platinum digital vernier calliper, which was printed on an A4 size paper. LDL band migration distances from the starting point for each sample were measured and converted to LDL size with a calibration curve plot obtained from three standards, apoferritin, thyroglobulin, and LDL. Based on Krauss et al., we set the limit for atherogenic LDL size as <25.5 nm [18]. Gel images of protein and lipid staining methods are presented in Figure 1.

HPLC method

The separation of lipoproteins by the HPLC method with gel permeation columns was developed by Hara and Okazaki in 1986 [20]. Shimadzu Prominence HPLC System (Shimadzu Inc., Japan) has two TSKgel LipopropakXL (300 × 7.8 mm, Tosoh) columns which are connected in tandem to provide higher resolution results with more discriminating power. The lipoproteins attached to the column are eluted in order of their size. The largest particles are released first (CM, VLDL), and the smallest is left last (HDL). After elution, lipoproteins are split and fed into two different reaction channels where enzymatic triglyceride and cholesterol measurements will be done. Signals received from two separate detectors are converted into the TC and TG chromatograms. For lipoproteins, the component peak analyses of four major classes (CM, VLDL, LDL, and HDL) and 20 subclasses were determined by the Gaussian curve fitting technique and the particle sizes of each lipoprotein were calculated by their elution times on a chromatogram. Latex beads of 10, 20, 25, 30, 35, 40, and 50 nm were used to plot a calibration curve and determine lipoprotein size. In this method, elution times for LDL peaks are seen approximately between 20 and 23 min. Particle sizes between 16 and 30 nm are defined as LDL. Major lipoproteins and 20 subclasses can be identified in less than 30 min. This HLPC procedure was performed in the laboratories of Skylight Biotech Company as described previously [15].

Statistical analysis

Statistical analysis was performed using Analyse-it software (Analyse-it Software, Ltd., Leeds, United Kingdom). Pearson correlation and Passing-Bablok regression analysis were performed to determine the

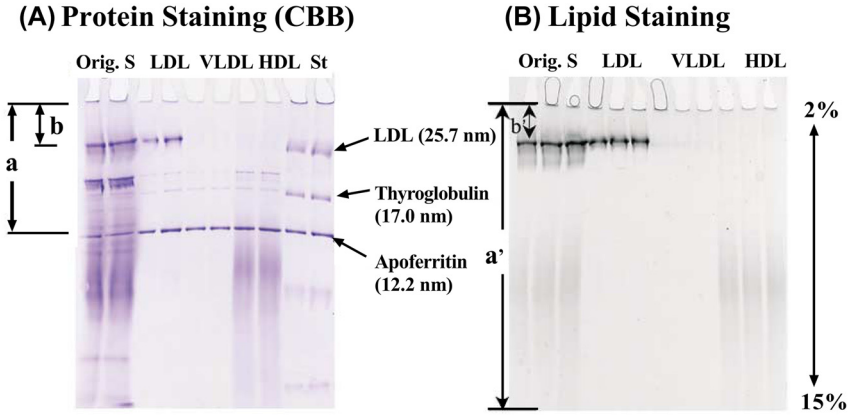

Figure 1: Comparison between protein staining and lipid staining of LDL particles obtained by 20–150 g/L PAGGE.
(A) Protein staining with Coomassie brilliant blue. LDL, VLDL, and HDL were isolated by equilibrium density gradient ultracentrifugation; a, distance from the starting point to apoferritin; b, distance from the starting point to whole-serum LDL. The original serum for preparation by ultracentrifugation was from a patient with type IIa hypercholesterolemia (TC 399 mg/dL, TG 32 mg/dL). (B) Lipid staining. Lipid staining of LDL was performed with Sudan black stain (5 g/L of SB in 60% methanol); a', distance from the starting point to the top of electrophoresis; b', distance from the starting point to the whole-serum LDL. St. Orig. S, original serum; St.LDL, low-density lipoprotein; St.VLDL, very low-density lipoprotein; St.HDL, high-density lipoprotein; St.TC, total cholesterol; St.TG, triglyceride.

strength of the linear relationship between the LDL size obtained by the two methods. In addition, the Bland-Altman plot was used to determine the mean difference and the agreement between the two methods was evaluated by Kappa analysis. According to previous studies, we received atherogenic LDL size as <25.5 nm [18]. The levels of LDL sizes obtained from both methods were expressed as mean ± standard deviation (SD).

Results

We summarise demographic information and lipid profile of the volunteers (n=87) from whom the samples were obtained in Table 1.

According to the correlation results, we determined a very strong correlation between all three LDL size methods (HPLC-TC and HPLC-TG r=0.941, p<0.001; HPLC-TC and PAGGE r=0.923, p<0.001; HPLC-TG and PAGGE r=0.910, p<0.001). In addition, we determined a significantly strong but negative correlation between triglyceride concentration and LDL size (for PAGGE r=−0.817, p<0.001; HPLC-TC r=−0.786, p<0.001 and HPLC-TG r=−0.789, p<0.001).

The LDL size means (95% confidence interval lower-upper limits) ± standard deviations obtained by PAGGE, HPLC-TC, and HPLC-TG methods were 25.77 (25.65–25.89) ± 0.54, 24.86 (24.74–24.98) ± 0.55, and 24.82 (24.72–24.94) ± 0.79 nm respectively. Comparison of the LDL size means among the methods were shown in Figure 2. The mean particle size of the LDL which was measured with the PAGGE method was found statistically higher than the other methods (p<0.001).

Table 1: Demographic information and lipid profile of volunteers (n=87).

Parameters	Mean	SD
Age, year	9.4	2.7
Gender[a], M/F	56/31	
BMI, kg/m^2	25.7	2.9
Weight status[a] (Normal/over-weight)	38/49	
Total cholesterol, mg/dL	170.8	34.5
HDL-C, mg/dL	48.3	8.6
LDL-C, mg/dL	98.9	33.6
Triglycerides, mg/dL	106.1	51.8
LDLs-PAGGE, nm	25.7	0.7
LDLs-HPLC-TC, nm	24.8	0.7
LDLs-HPLC-TG, nm	24.8	0.8

[a]given as count (n). According to BMI: normal <25, over-weight ≥25. BMI, body mass index; F, female; M, male; LDLs, LDL size; SD, standard deviation; HPLC-TC, high-performance liquid chromatography-total cholesterol; HPLC-TG, high-performance liquid chromatography-triglyceride; PAGGE, polyacrylamide gradient gel electrophoresis.

In the Passing-Bablok regression analysis for PAGGE and HPLC-TC, the estimated intercept, slope, and 95% confidence intervals were 0.90 (0.66–3.01) and 1.0 (0.91–1.01) respectively (Figure 3A). For the PAGGE and HPLC-TG, the estimated intercept, slope, and 95% confidence intervals were 5.56 (3.17–7.73) and 0.81 (0.72–0.91) respectively (Figure 3B).

In the Bland-Altman plot, we determined the mean difference as 0.91 (0.85–0.97) nm between PAGGE and

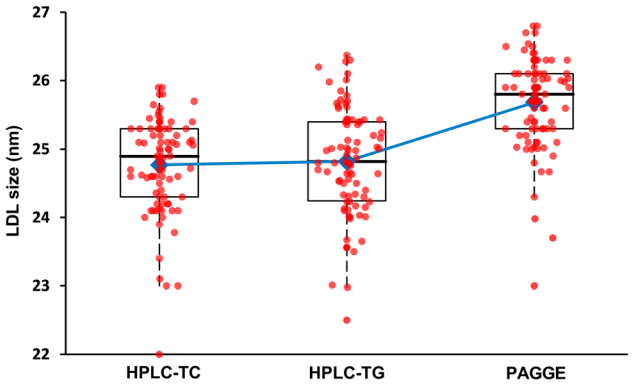

Figure 2: The Box plot presentation of LDL size levels.
The blue squares represent the means and black lines represent the median values of LDL size determined by methods. HPLC-TC, high-performance liquid chromatography-total cholesterol; HPLC-TG, high-performance liquid chromatography-triglyceride; PAGGE, polyacrylamide gradient gel electrophoresis.

HPLC-TC. The mean difference between PAGGE and HPLC-TG was determined as 0.87 (0.80–0.93) nm. In addition, we determined the limit of agreement (LoA) and 95% confidence intervals of LoA which are shown in Figure 3C and D.

To calculate the within-day precision (CVw) of the PAGGE method, a pooled sample was analysed consecutively 20 times in a run. For between-day precision (CVb), the same sample was analysed in duplicate for 20 consecutive days. Imprecision determined with a single level sample was found for CVw as 2%, and CVb as 3%.

When we assessed the compatibility of the methods according to the atherogenic LDL size (<25.5 nm) [18] the highest agreement was found between HPLC-TG and HPLC-TC methods. In addition, the kappa value for HPLC-TG-PAGGE was higher than those of the HPLC-TC-PAGGE (Table 2).

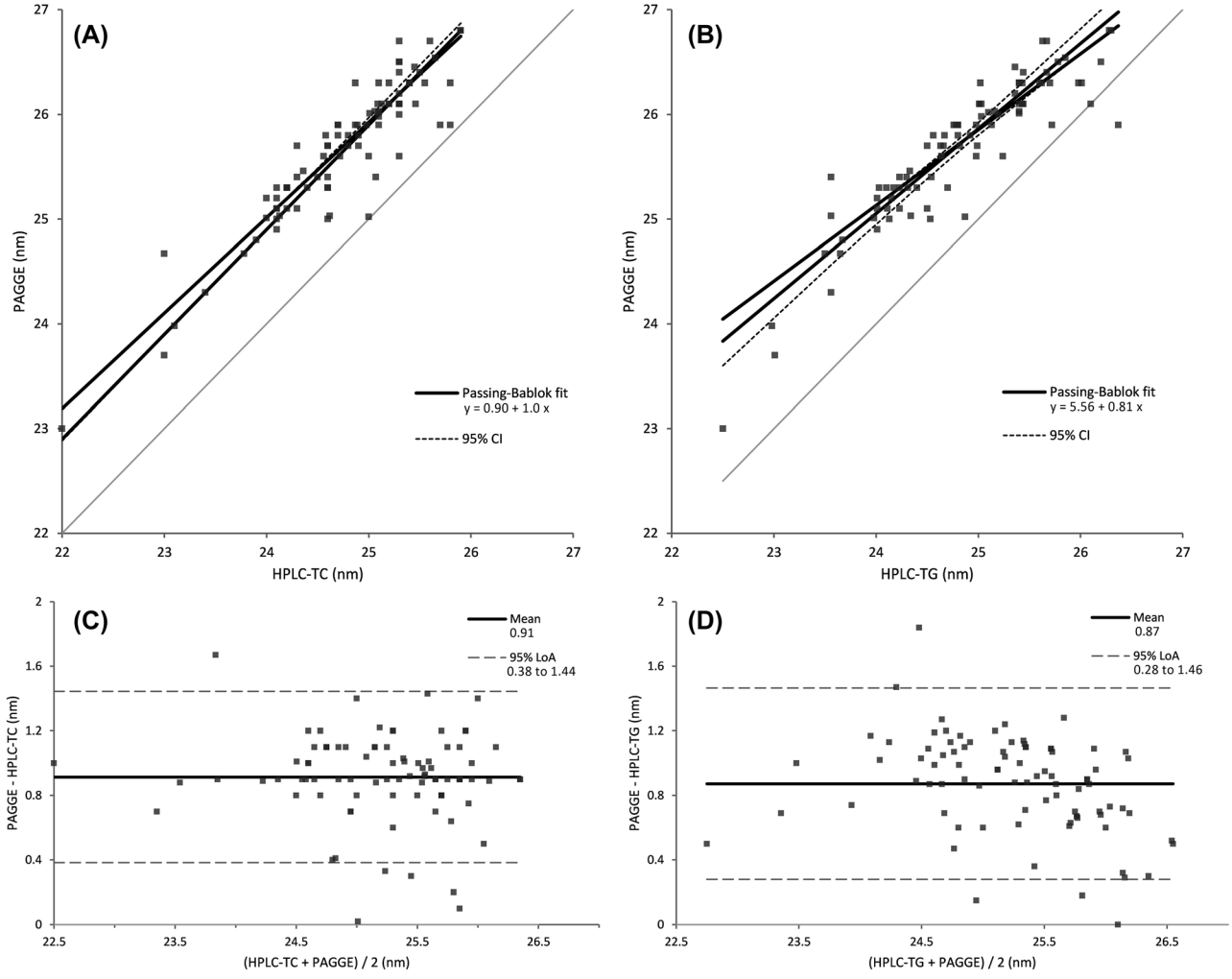

Figure 3: Passing-Bablok regression analysis (A and B) and Bland-Altman plots (C and D).
HPLC-TC, high-performance liquid chromatography-cholesterol; HPLC-TG, high-performance liquid chromatography-triglyceride; PAGGE, polyacrylamide gradient gel electrophoresis.

Table 2: The kappa values and agreement between the methods.

	Kappa (CI 95%)	Agreement (CI 95%)
HPLC-TC- HPLC-TG	0.68 (0.46–0.96)	92.0 (84.3–96.0)
HPLC-TC- PAGGE	0.44 (0.20–0.69)	85.1 (76.1–91.1)
HPLC-TG- PAGGE	0.71 (0.52–0.90)	90.8 (82.9–95.3)

CI, confidence interval; HPLC-TC, high-performance liquid chromatography-total cholesterol; HPLC-TG, high-performance liquid chromatography-triglyceride; PAGGE, polyacrylamide gradient gel electrophoresis.

Discussion

In this study, we compared the modified Krauss PAGGE and HPLC methods in determining LDL size. We found that the PAGGE method yielded results comparable with the HPLC method in the determination of LDL size.

LDL size has been demonstrated to be associated with the risk of developing cardiovascular disease [21, 22]. In a prospective population-based study, a smaller LDL size was reported to be the best predictor of coronary artery disease [23]. Therefore, determining LDL size timely is important to evaluate the risk for and to manage cardiovascular disease. Currently used methods for determining LDL size and separating LDL subfractions including ultracentrifugation, HPLC, NMR spectroscopy, and electrophoresis. However, these methods are time-consuming, labour-intensive, require experienced staff, and expensive instrumentation. Today, many clinical laboratories need a simple, easy to perform, inexpensive, and proven method for measuring LDL size. Studies have been conducted to compare the effectiveness of various methods used for determining LDL size [12, 14, 24]. Hoefner et al. compared polyacrylamide tube gel electrophoresis (PTGE) and NMR spectroscopy methods in 51 samples and reported a concordance of 76% [24].

In the present study, we compared the PAGGE method with the HPLC method using cholesterol peak and triglyceride peak. The method originally described by Krauss and Burke in 1982 for measuring LDL size is not easy to use today [18]. Pharmacia 2/16 gel used in the original method is not produced anymore. In our study, we used 20–150 g/L polyacrylamide gel. The original method has two steps. In the first step, 24-h polyacrylamide gel electrophoresis is applied so that the negative charges of free fatty acids in the samples with hypertriglyceridemia do not interfere with electrophoresis [25]. LDL size measurement gives wrong results in the presence of negative charges when standard polyacrylamide gel electrophoresis (PAGE) which has a uniform gradient is used. Therefore, in our study, we used 2/15 polyacrylamide gel to overcome this weakness of standard PAGE methods. In the second step, Sudan Black staining was used for lipoproteins, while protein staining was performed in the standards and latex control pieces in the original Krauss method. Differently, we added apoferritin to all samples as the internal standard and stained LDL lipoproteins with CBB. The two-step staining procedure in the original method was reduced to a single-step protein staining in the modified method. In addition, since apoferritin is added to all samples as an internal standard and stained together with lipoproteins, they can be evaluated in the same gel image. In this way, the performance improvement was achieved by the quality problems related to the method were resolved.

In the HPLC method, it is still not clear which of the results of the two different methods, including HPLC-TC and HPLC-TG, given for the LDL size will be used [14]. Although the two different LDL sizes under the cholesterol and triglyceride curves seem to be compatible with each other, using a single LDL size result will reduce confusion. It is important for the compatibility of the methods that the 95% confidence intervals of the intercept and the slope detected in the Passing-Bablok analysis encompass 0 and 1, respectively. In the comparison of LDL sizes obtained by PAGGE and HPLC-TC methods, while the condition mentioned above was met for the slope obtained from the Passing-Bablok regression analysis, it was not valid for the intercept. However, since the confidence interval of the intercept is very close to zero (0.66–3.01), we consider that both methods can be used interchangeably. In the comparison of LDL sizes obtained by PAGGE and HPLC-TG methods, neither slope nor intercept met the requirements. These results show that the agreement of both methods is lower. In addition, these results were also confirmed with the Bland-Altman analysis.

We evaluated the consistency between methods according to the definition of atherogenic LDL (<25.5 nm) by Kappa analysis. The highest kappa value was determined for HPLC-TG- PAGGE, while the highest agreement value was determined for HPLC-TC- HPLC-TG. These results support the regression and Bland-Altman results.

In our study, imprecision was evaluated with within-day and between-day precision measurements. Accordingly, within-day precision (CVw) was found as 2% and between-day (CVb) as 3%. These results suggest a good imprecision and reproducibility of the PAGGE method. HPLC method, for LDL size, within-day precision was found as <4.7% for under cholesterol curve and <4.2% for under triglycerides curve [14]. Interestingly, the same study was achieved a better between-day precision as <3.3% for under cholesterol curve and <2.5% for under triglycerides curve [14]. Although we did not measure precision with the

HPLC method, our results of the PAGGE method are lower than that study.

Study limitations

A reliable comparison could not be made between studies due to the differences in the methods used in LDL size measurement. It is difficult to find common ground between the methods. In addition, we could not analyse the imprecision of the HPLC method. In addition, it would be appropriate to study in groups with higher LDL sizes. It is also necessary to carry out studies to determine the cut-off value for the LDL size.

Conclusions

The results of this study indicate that the PAGGE method can be used as a reliable method for measuring LDL size. HPLC methods under cholesterol and triglycerides may be used interchangeably, but with different clinical decision limits. However, the results of this study should be confirmed with further comprehensive studies. In addition, our study demonstrated that the measurement methods for LDL size could be simplified with several modifications like just protein staining and adding apoferritin internal standard. Therefore, these methods are open for further research on refinements and comparison.

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Competing interests: Authors reported no conflict of interest to disclose.

Informed consent: Informed consent was obtained from all individual participants included in the study.

Ethical approval: The study was approved by the Gulhane Military Medical Committee Academic Board (dated September 26, 2005, decision no: 34) following the ethical standards established by the institution, and all procedures were applied in accordance with the Helsinki Declaration.

Data availability: Data used in this study are included in the manuscript.

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