



Case Report

Evaluation of a gel-permeation high-performance liquid chromatography for determining triglyceride levels in serum major lipoproteins, compared with the ultracentrifugation/precipitation method

Shinichi Usui^a, Tetsuya Mizuno^b, Mitsuyo Okazaki^{b,*},
Masakazu Nakamura^c, Ikunosuke Sakurabayashi^d

^a Department of Medical Technology, Okayama University Graduate School of Health Sciences, Okayama, Japan

^b College of Liberal Arts and Sciences, Tokyo Medical and Dental University, 2-8-30, Kohnodai, Ichikawa-shi, Chiba 272-0827, Japan

^c Osaka Medical Center for Health Science and Promotion, Osaka, Japan

^d Jichi Medical University, Saitama, Japan

Received 11 June 2008; received in revised form 15 October 2008; accepted 15 October 2008

Available online 25 October 2008

Abstract

Objectives: To evaluate the gel permeation high-performance liquid chromatography (GP-HPLC) method for determination of triglyceride (TG) levels in low-density lipoprotein (LDL) and high-density lipoprotein (HDL).

Design and methods: The GP-HPLC and the ultracentrifugation(UC)/precipitation methods were used and compared.

Results: There was no significant difference in measured levels of LDL-triglyceride between the two methods, but the HDL-triglyceride levels measured by the GP-HPLC technique were significantly higher than the UC/precipitation one (145 ± 47 mg/L and 121 ± 45 mg/L respectively, $n=38$, $p<0.0001$).

Conclusions: A GP-HPLC technique provides LDL-TG levels comparable to those obtained by the UC/precipitation method.

© 2008 The Canadian Society of Clinical Chemists. Published by Elsevier Inc. All rights reserved.

Keywords: HPLC; Triglyceride; Cholesterol; Lipoprotein

Introduction

A gel-permeation high-performance liquid chromatography (GP-HPLC) is a method for classifying lipoproteins on the basis of their particle sizes [1,2]. Major classes and subclasses of lipoproteins have been defined by component peak analyses on the basis of lipoprotein particle sizes by Gaussian curve fitting technique [2–4]. In our GP-HPLC method, the splitting of the column effluent equally into two lines allows simultaneous measurement of cholesterol and triglyceride (TG) levels in lipoproteins separated by gel permeation columns [5], and this

system is used widely for lipoprotein profiles for non-human being as well as human subjects [6,7].

We previously reported good traceability of GP-HPLC to the reference methods, Abell-Kendall for total cholesterol (TC), the ultracentrifugation(UC)/precipitation (heparin-MnCl₂) method for high-density lipoprotein cholesterol (HDL-C) [8], and the beta-quantification method for low-density lipoprotein cholesterol (LDL-C) levels [9]. These evaluations of analytical accuracy for the GP-HPLC method have been limited in cholesterol measurement, and no information is available on TG measurement.

The aim of this study was to compare the GP-HPLC method with the UC/precipitation method, focusing on LDL-TG and HDL-TG determination in 38 male firefighters. Moreover we discussed the significance of TG measurements by investigating the relationship between cholesterol and TG levels in major lipoproteins and their subclasses.

Abbreviations: GP-HPLC, gel-permeation high-performance liquid chromatography; TG, triglyceride; TC, total cholesterol; UC, ultracentrifugation; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; VLDL, very low-density lipoprotein; CM, chylomicron.

* Corresponding author.

E-mail address: okazaki.las@tmd.ac.jp (M. Okazaki).

Materials and methods

Serum samples

We obtained blood samples from 38 male firefighters (aged 19–54) at Kashiwa firehouse, Chiba, Japan, participating in a study of health care and physical fitness [10]. The blood samples were collected into glass tubes without anticoagulant after fasting of 16 h, allowed to clot at room temperature and centrifuged at 2000 g for 15 min to obtain serum samples. All these serum samples were stored at 4 °C and analyzed within 5 days after blood collection. All volunteers gave informed consent to participate in this study, as stated in the declaration of Helsinki [11].

Lipoprotein analysis by a dual detection GP-HPLC system

Serum lipoproteins were analyzed by analytical HPLC service system ([®]LipoSEARCH) at Skylight Biotech Inc. (Akita, Japan), as previously described [5]. In brief, a 10 µL of whole serum sample was injected into two connected columns (300×7.8 mm) of TSKgel LipopropakXL (Tosoh, Tokyo, Japan) and lipoproteins were separated with 0.05 mol/L Tris-buffered acetate (pH 8.0) containing 0.3 mol/L sodium acetate, 0.05% sodium azide, and 0.005% Brij-35 at a flow rate of 0.7 mL/min. The column effluent was split equally into two lines by a P-460 MicroSplitter (Upchurch Scientific Inc., Oak Harbor, WA); one effluent portion was mixed with cholesterol reagent (Determiner L TC/Kyowa Medex, Tokyo) and the other with TG reagent (Determiner L TG/Kyowa Medex). The two enzymatic reagents were each pumped at a flow rate of 0.35 mL/min. The absorbance at 550 nm was continuously monitored after the enzymatic reaction at 37 °C in a reactor coil [Teflon; 15 m×0.4 mm (i.d.)].

We defined 3 very low-density lipoprotein (VLDL) subclasses, 4 LDL subclasses, and 5 HDL subclasses using 20 component peaks on the basis of lipoprotein particle size (diameter), as described previously [2–4]. In this study, chylomicron (CM) was included in the VLDL fraction, because of a very small amount of CM detected (0.06±0.07 mg/L for cholesterol and 1.7±2.1 mg/L for TG).

The ultracentrifugation/precipitation method

Determination of TG levels was carried out in lipoprotein fractions that were separated according to modified procedures of the beta-quantification method for LDL-C [12] and the designated comparison method for HDL-C [13,14]. Briefly, a 1.0-mL serum sample was placed in a polycarbonate centrifuge tube (cat. no. 343778; Beckman Coulter, Inc., Fullerton, CA) and centrifuged on a Beckman Optima-TLX preparative ultracentrifuge with a fixed-angle TLA 120.2 rotor at 527 000 g for 3 h at 16 °C, to obtain a bottom ($d > 1.006$ kg/L) fraction including LDL and HDL. A HDL fraction was separated directly from whole serum, where apolipoprotein B-containing lipoproteins were precipitated with Dextran-sulfate/MgCl₂. TG concentrations of whole serum, the LDL+HDL fraction

($d > 1.006$ kg/L), and the HDL fraction (Dextran-sulfate/MgCl₂ supernatant) were determined enzymatically on the Hitachi 7170 S automated analyzer with Determiner L TG II (Kyowa Medex). The levels of LDL-TG were calculated by subtracting HDL-TG values from TG concentration in the HDL+LDL fraction ($d > 1.006$ kg/L). The levels of VLDL-TG were calculated by subtracting TG concentrations in the HDL+LDL fraction from serum TG levels. Cholesterol levels of each fraction were also similarly determined by Determiner L TC II (Kyowa Medex), followed by the same calculation for each lipoprotein class.

Statistical analysis

Data were analyzed with SPSS Version 10.0 (SPSS Inc, Chicago, IL). Student's *t* test was performed to compare the differences between two sample means. The results are expressed as mean±standard deviation. All the reported *p* values were 2-tailed, and those <0.05 were considered to be statistically significant. Correlations between various variables are presented as the Pearson's correlation coefficient (*r*-value).

Results

Analytical precision for the dual GP-HPLC detection system

Within-run reproducibility (*n*=5) of the dual GP-HPLC system for TC and TG measurements was determined on two different pooled serum samples. Pool 1, a serum-based frozen material (Kyowa Medex, Japan), is normolipidemic (TC=1530 mg/L, TG=710 mg/L), and Pool 2, a pool of sera obtained from 3 laboratory volunteers, is a hyperlipidemic aliquot (TC=1630 mg/L, TG=4440 mg/L). Good within-run imprecision values were obtained in major lipoproteins and their subclasses, and coefficients of variation were less than 1.82% except for CM.

Quantitative comparison of major lipoproteins assayed by the UC/precipitation and the GP-HPLC methods

There was no significant difference in the average values of serum TG levels between the GP-HPLC (1138±657 mg/L) and the automated enzymatic method (1128±652 mg/L). Shown in Table 1 are the results of cholesterol and TG levels in major lipoproteins and the TG/cholesterol ratios, determined by the UC/precipitation and the GP-HPLC methods.

As for the VLDL and LDL fractions, TG levels determined by the GP-HPLC were correlated significantly with those by the UC/precipitation method, and significant differences were not found. The VLDL-TG/VLDL-C ratio was significantly lower in the GP-HPLC method, although a very high correlation was found between the two methods.

In contrast to VLDL and LDL fractions, the HDL fraction was significantly different in HDL-C and HDL-TG levels and the HDL-TG/HDL-C ratios, although high correlations were obtained between the two methods.

Table 1

Comparison of TG and cholesterol levels in major lipoprotein classes between the GP-HPLC and the UC/precipitation methods ($n=38$)

Variable	UC/precipitation method		GP-HPLC method		Correlation		Paired <i>t</i> test
	Mean \pm SD	Range	Mean \pm SD	Range	<i>r</i>	<i>p</i> value	<i>p</i> value
TG (mg/L)	1128 \pm 652	410–3410	1138 \pm 657	425–3558	0.995	0.000	0.330
TC (mg/L)	1961 \pm 350	1220–2720	1980 \pm 347	1227–2680	0.989	0.000	0.032
VLDL-TG (mg/L)	709 \pm 552	130–2340	692 \pm 518	198–2443	0.989	0.000	0.207
VLDL-C (mg/L)	214 \pm 129	30–620	238 \pm 122	56–557	0.827	0.000	0.053
VLDL-TG/VLDL-C ratio	3.45 \pm 1.71	0.94–8.06	2.80 \pm 0.91	0.99–4.50	0.539	0.000	0.008
LDL-TG (mg/L)	297 \pm 136	164–894	302 \pm 148	127–893	0.931	0.000	0.620
LDL-C (mg/L)	1202 \pm 339	573–1815	1155 \pm 315	581–1699	0.982	0.000	0.000
LDL-TG/LDL-C ratio	0.25 \pm 0.10	0.14–0.59	0.26 \pm 0.10	0.14–0.62	0.887	0.000	0.243
HDL-TG (mg/L)	121 \pm 45	55–220	145 \pm 47	73–265	0.970	0.000	0.000
HDL-C (mg/L)	546 \pm 117	330–748	587 \pm 120	367–790	0.992	0.000	0.000
HDL-TG/HDL-C ratio	0.24 \pm 0.11	0.07–0.48	0.26 \pm 0.11	0.10–0.53	0.983	0.000	0.000

GP-HPLC, gel permeation high-performance liquid chromatography; UC, ultracentrifugation; TG, triglyceride; TC, total cholesterol; VLDL-TG, very low-density lipoprotein triglyceride; VLDL-C, very low-density lipoprotein cholesterol; LDL-TG, low-density lipoprotein triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-TG, high-density lipoprotein triglyceride; HDL-C, high-density lipoprotein cholesterol.

Relationship between TG and cholesterol levels in major lipoproteins and their subclasses

We examined the relation between TG and cholesterol levels in major lipoprotein classes and their subclasses as shown in Table 2. Serum TG levels had significant positive or negative correlations with cholesterol levels in all major lipoproteins and most of their subclasses. Therefore, partial correlation analyses by adjustment for serum TG levels are carried out, as shown in Table 2.

The VLDL-TG and the LDL-TG levels, like serum TG levels, were almost similarly associated with cholesterol levels of some lipoprotein subclasses. The adjustment for serum TG levels, however, did not remove the significant correlations between the VLDL-TG and the large VLDL-C levels. The significant correlations of the LDL-TG levels with total LDL-C and several lipoprotein subclasses (large VLDL-C, small VLDL-C, small LDL-C, and very small LDL-C) also remained after adjustment for serum TG levels. HDL-TG levels showed significant positive correlations with total VLDL and all of the VLDL subclasses but no correlation with total LDL, total HDL and their subclasses except for very small LDL-C.

Discussion

In our GP-HPLC method using the dual detection system by splitting the column effluent into two lines, cholesterol and TG levels in VLDL, LDL and HDL and their subclasses can be obtained by applying 10 μ L of whole serum or plasma at the interval of 24 min. The 20 component peak separation by Gaussian curve fitting technique could determine cholesterol levels in major lipoprotein classes and their subclasses as described previously [2–4,15].

In this study, the same lipoprotein definition of 20 component peaks for cholesterol profile was applied to the TG profile, and the results were compared with those by the UC/precipitation method. Similarly to cholesterol measurements, there were high correlations between the two methods in TG measurements, but HDL-TG levels were systematically higher in the GP-HPLC than the UC/precipitation method, as

observed in HDL-C measurements. We found no effects of serum TG and lipoprotein(a) levels on the HDL-TG measurements (data not shown), and slightly but significantly higher

Table 2

Correlations of TG with cholesterol levels in lipoprotein classes measured by the GP-HPLC method ($n=38$)

Lipoprotein classes	TG	VLDL-TG	LDL-TG	HDL-TG
VLDL-C	0.875 ^a	0.849 ^a (−0.018)	0.769 ^a (0.121)	0.523 ^a (0.126)
Large VLDL-C	0.933 ^a	0.946 ^a (0.466) ^b	0.665 ^a (−0.623) ^a	0.358 ^c (−0.479) ^c
Medium VLDL-C	0.766 ^a	0.733 ^a (−0.075)	0.694 ^a (0.141)	0.520 ^a (0.198)
Small VLDL-C	0.443 ^b	0.389 ^c (−0.201)	0.560 ^a (0.387) ^c	0.442 ^b (0.269)
LDL-C	0.515 ^a	0.452 ^b (−0.248)	0.598 ^a (0.355) ^c	0.099 (−0.248)
Large LDL-C	0.097	0.055 (−0.168)	0.227 (0.272)	0.003 (−0.059)
Medium LDL-C	0.318	0.254 (−0.247)	0.415 ^b (0.289)	0.005 (−0.208)
Small LDL-C	0.634 ^a	0.567 ^a (−0.277)	0.684 ^a (0.358) ^c	0.162 (−0.277)
Very small LDL-C	0.742 ^a	0.697 ^a (−0.156)	0.760 ^a (0.373) ^c	0.184 (−0.383) ^c
HDL-C	−0.640 ^a	−0.615 ^a (0.044)	−0.494 ^b (0.112)	−0.271 (0.114)
Very large HDL-C	−0.099	−0.094 (0.010)	0.052 (0.253)	−0.244 (−0.227)
Large HDL-C	−0.682 ^a	−0.670 ^a (−0.039)	−0.510 ^b (0.164)	−0.274 (0.153)
Medium HDL-C	−0.591 ^a	−0.579 ^a (−0.024)	−0.453 ^b (0.105)	−0.133 (0.273)
Small HDL-C	−0.167	−0.105 (0.251)	−0.194 (−0.101)	−0.180 (−0.108)
Very small HDL-C	0.020	−0.301 (0.003)	0.013 (−0.001)	−0.060 (−0.002)

Partial correlation coefficients after adjustment for serum TG are presented in parentheses.

Abbreviations are the same as indicated in Table 1.

^a $p < 0.001$.

^b $p < 0.01$.

^c $p < 0.05$.

HDL-TG/HDL-C ratios were obtained by the GP-HPLC method. These results may indicate an over-precipitation of TG-rich HDL particles by the precipitation reagent, but it was unclear where the precipitated HDL should be recovered because of a good agreement observed between the two methods in the LDL-TG and the VLDL-TG determinations.

We examined a significance of TG measurements of major lipoprotein classes using the correlation analyses (Table 2). Our findings show that the levels of serum TG were positively correlated with VLDL-C and negatively with HDL-C. Generally, most of serum TG are carried in VLDL (and CM after meal), and correlated well with VLDL-C levels. Indeed, VLDL-C can be estimated from measured serum TG values divided by 5, as used in the Friedewald equation [16]. In addition, there is commonly a metabolic relationship between serum TG and HDL-C [17–19]. We found significant negative correlations of HDL-C and their subclass with VLDL-TG and LDL-TG as well as serum TG, but these relationships disappeared after adjustment for serum TG levels, indicating that no additional information and advance are obtained from the measurements of VLDL-TG and LDL-TG levels. Earlier studies, however, reported high LDL-TG levels are indicative of cholesterol ester-depleted LDL, elevated intermediate-density lipoprotein, and dense LDL [20,21]. Consistent with these data, our results similarly indicates increased levels of LDL-TG are associated with the increased levels of small VLDL, small LDL, and very small LDL, independently of serum TG levels.

It is interesting that HDL-TG levels were not associated with HDL-C levels, because TG contents are usually increased or decreased in parallel with cholesterol contents, as observed in VLDL and LDL fractions. TG enrichment of HDL, which often occurs in hypertriglyceridemic states, significantly enhances the clearance of HDL apolipoprotein A-I from the circulation of healthy humans [18,19]. Therefore, measurements of HDL-TG and the HDL-TG/HDL-C ratio may provide an additional clinical tool for assessment of individuals and have greater ability to predict coronary heart disease risk than HDL-C itself.

In conclusion, a very simple and rapid GP-HPLC technique was confirmed to give TG levels in LDL, which were comparable to those obtained by the UC/precipitation method. Further study will be required to establish a clinical utility of measuring TG levels in each of major lipoproteins.

Acknowledgments

We gratefully acknowledge Kyowa Medex, Japan for providing the enzyme reagents for the cholesterol and the TG measurements by GP-HPLC. We greatly thank Skylight Biotech Inc. (Akita, Japan) for the technical assistance in this study.

References

- [1] Hara I, Okazaki M. High-performance liquid chromatography of serum lipoproteins. *Methods Enzymol* 1986;129:57–78.
- [2] Okazaki M, Usui S, Hosaki S. Analysis of plasma lipoproteins by gel permeation chromatography. In: Rifai N, Warnick GR, Dominiczak MH, editors. Handbook of lipoprotein testing. Washington DC: AACC Press; 2000. p. 647–69.
- [3] Okazaki M, Usui S, Ishigami M, et al. Identification of unique lipoprotein subclasses for visceral obesity by component analysis of cholesterol profile in HPLC. *Arterioscler Thromb Vasc Biol* 2005;25:578–84.
- [4] Okazaki M, Usui S, Fukui A, Kubota I, Tomoike H. Component analysis of HPLC profiles of unique lipoprotein subclass cholesterol for detection of coronary artery disease. *Clin Chem* 2006;52:2049–53.
- [5] Usui S, Hara Y, Hosaki S, Okazaki M. A new on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides in lipoproteins by HPLC. *J Lipid Res* 2002;43:805–14.
- [6] Kawai T, Ito T, Ohwada K, Mera Y, Matsushita M, Tomoike H. Hereditary postprandial hypertriglyceridemic rabbit exhibits insulin resistance and central obesity: a novel model of metabolic syndrome. *Arterioscler Thromb Vasc Biol* 2006;26:2752–7.
- [7] Hu W, Abe-Dohmae S, Tsujita M, et al. Biogenesis of HDL by SAA is dependent on ABCA1 in the liver in vivo. *J Lipid Res* 2008;49:386–93.
- [8] Usui S, Nakamura M, Jitsukata K, Nara M, Hosaki S, Okazaki M. Assessment of between-instrument variations in a HPLC method for serum lipoproteins and its traceability to reference methods for total cholesterol and HDL-cholesterol. *Clin Chem* 2000;46:63–72.
- [9] Okazaki M, Usui S, Nakamura M, Yamashita S. Evaluation of HPLC method for LDL-C determination in lipoprotein abnormalities, comparing to the reference method of beta-quantification. *Clin Chim Acta* 2008;395: 62–7.
- [10] Mizuno T, Yamami N, Shibayama M, Mano Y, Fukubayashi T. Study on health care and physical fitness program of Japanese fire-fighters. 52nd meeting of the Japanese Society of Physical Education, Sapporo, Japan; 2001.
- [11] Carlson RV, Boyd KM, Webb DJ. The revision of the Declaration of Helsinki: past, present and future. *Br J Clin Pharmacol* 2004;57:695–713.
- [12] Myers GL, Cooper GR, Greenberg N, Kimberly MM, Waymack PP, Hassemér DJ. Standardization of lipid and lipoprotein measurements. In: Rifai N, Warnick GR, Dominiczak MH, editors. Handbook of lipoprotein testing. 2nd ed. Washington, DC: AACC Press; 2000. p. 717–48.
- [13] Wu LL, Warnick GR, Wu JT, Williams RR, Lalouel JM. A rapid micro-scale procedure for determination of the total lipid profile. *Clin Chem* 1989;35:1486–94.
- [14] Esteban-Salañ M, Guimon-Bardesi A, de La Viuda-Unzueta JM, Azcarate-Ania MN, Pascual-Usandizaga P, Amoroto-Del-Rio E. Analytical and clinical evaluation of two homogeneous assays for LDL-cholesterol in hyperlipidemic patients. *Clin Chem* 2000;46:1121–31.
- [15] Kazama H, Usui S, Okazaki M, Hosoi T, Ito H, Orimo H. Effects of bezafibrate and pravastatin on remnant-like lipoprotein particles and lipoprotein subclasses in type 2 diabetes. *Diabetes Res Clin Pract* 2003;59:181–9.
- [16] Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972;18:499–502.
- [17] Cullen P. Evidence that triglycerides are an independent coronary heart disease risk factor. *Am J Cardiol* 2000;86:943–9.
- [18] Rashid S, Watanabe T, Sakaue T, Lewis GF. Mechanisms of HDL lowering in insulin resistant, hypertriglyceridemic states: the combined effect of HDL triglyceride enrichment and elevated hepatic lipase activity. *Clin Biochem* 2003;36:421–9.
- [19] Lamarche B, Uffelman KD, Carpentier A, et al. Triglyceride enrichment of HDL enhances in vivo metabolic clearance of HDL apo A-I in healthy men. *J Clin Invest* 1999;103:1191–9.
- [20] Ruotolo G, Ericsson CG, Tettamanti C, et al. Treatment effects on serum lipoprotein lipids, apolipoproteins and low density lipoprotein particle size and relationships of lipoprotein variables to progression of coronary artery disease in the Bezafibrate Coronary Atherosclerosis Intervention Trial (BECAIT). *J Am Coll Cardiol* 1998;32:1648–56.
- [21] März W, Scharnagl H, Winkler K, et al. Low-density lipoprotein triglycerides associated with low-grade systemic inflammation, adhesion molecules, and angiographic coronary artery disease: the Ludwigshafen Risk and Cardiovascular Health study. *Circulation* 2004;110:3068–74.