Chemistry and Physics of Lipids 165 (2012) 133–141

Contents lists available at SciVerse ScienceDirect

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip

A simple and sensitive method for lipoprotein and lipids profiles analysis of individual micro-liter scale serum samples

Liu Yang, Baoyan Fan, Kangmin Yang, Haibo Zhu*

State Key Laboratory for Bioactive Substances and Functions of Natural Medicines & Ministry of Health Key Laboratory of Biosynthesis of Natural Products, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, PR China

ARTICLE INFO

Article history:
Received 3 October 2011
Received in revised form
22 November 2011
Accepted 23 November 2011
Available online 1 December 2011

Keywords:
Lipoprotein
Lipids
FPLC
Superdex 200 10/300GL
Amplex Red
Nile Red
Dyslipoproteinemia
Atherosclerosis

ABSTRACT

A simple and sensitive method to determine lipoprotein and lipids profiles in micro-liter scale individual serum sample is not presently available. Traditional lipoprotein separation techniques either by ultra-centrifugation or by liquid chromatography methods have their disadvantages in both lipoprotein separation and lipids component quantification. In this study we used small volume needing size-exclusion fast protein liquid chromatography to separate different lipoprotein subclasses in 50 μL serum. And lipids contents, such as cholesterol, cholesterol ester and triacylglycerol, were measured by using two different fluorescence-based lipid detection methods. With this method, very low density lipoprotein, low density lipoprotein and high density lipoprotein could be easily separated, and follow-up lipid detection was completed by simple kinds of reactions. Serum lipoprotein and lipids profiling from C57BL/6 mice ($n = 5$) and human ($n = 5$) were analyzed. The elution profiles of five individuals were highly reproducible, and there were lipoprotein and lipids distribution variations between C57BL/6 mice and human beings. In conclusion, this method which combined small volume needing size-exclusion fast protein liquid chromatography and fluorescence-based lipids measurement, provided a simple, efficient, integrity and reproducible procedure for determining serum lipoprotein and lipids profiles in micro-liter scale levels. It becomes possible that determination of lipoprotein profiles and gaining information of lipids in different lipoproteins can be accomplished simultaneously.

1. Introduction

A lipoprotein is a biochemical assembled protein and lipid complex (Olson, 1998), which enables hydrophobic lipids to be carried in the aqueous blood stream and participate in energy material supply and lipids metabolism. The proteins in lipoprotein include different kinds of apolipoprotein, enzymes, coenzymes, transporters and complement components (Vaisar et al., 2007). The major lipid components involved in the complex are cholesterol (C), cholesteryl esters (CE), triacylglycerol (TAG) and phospholipids (PL). Lipoproteins are sub-classified by different density, ranging as very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) (Ordovas and Osgood, 1998), which are different in apolipoprotein composition, lipid component, size, charge, and physiological functions (Collins et al., 2010). The different roles of these lipoproteins in the lipid metabolism and the development of atherosclerosis have attracted scientist's attention. Many studies were made to find the relationships among lipoprotein, lipoprotein metabolism associated enzymes and lipoprotein metabolism disorder related diseases.

There are several methods available for isolation of plasma lipoprotein, such as paper and agarose electrophoresis (Cooper and Mandel, 1954), non-denaturing polyacrylamide gradient gel electrophoresis (Krauss and Burke, 1982; Li et al., 1994; McNamara et al., 1996), ultra-centrifugation (Havel et al., 1955; Grundy, 2004), immunoseparation (Zilversmit, 1973, 1995; Tatami et al., 1981) and chromatography (Teizo Sata et al., 1972; Okazaki and Hara, 1980). Of which, the most commonly used is the ultra-centrifugation, but the time consuming and large sample volumes requiring restrict its application for the individual small volume serum, and the high g-forces and salt concentrations of the medium required for this traditional technique may cause structural changes and dissociation of lipoprotein (Kunitake and Kane, 1982; Innis-Whitehouse

Abbreviations: Apo AI, Apolipoprotein AI; Apo B, Apolipoprotein B; Apo CIII, Apolipoprotein CIII; AV, Average; C, Cholesterol; CE, Cholesteryl esters; FPLC, Fast protein liquid chromatography; HDL, High density lipoprotein; LDL, Low density lipoprotein; PL, Phospholipids; R_{St}, Stoke's radius; SEC, Size exclusion chromatography; SD, Standard deviation; TAG, Triacylglycerol; TC, Total cholesterol; VLDL, Very low density lipoprotein; V_e, Elution volume.

* Corresponding author at: State Key Laboratory for Bioactive Substances and Functions of Natural Medicines & Ministry of Health Key Laboratory of Biosynthesis of Natural Products, Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Nan Wei Road A2, Beijing 100050, PR China. Tel.: +86 10 63188106; fax: +86 10 6307757.
E-mail addresses: zhuhaibo@imm.ac.cn, yangliu@imm.ac.cn (H. Zhu).

0009-3084/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.chemphyslip.2011.11.010

et al., 1998). Fast protein liquid chromatography (FPLC) is a rapidly developed technique that has been validated for the isolation of lipoprotein from relative small sample volumes (März et al., 1993; Garber et al., 2000), the size-exclusion chromatography (SEC) separation and physiological mimetic elution condition assure the structure and component integrity of separated lipoprotein classes. After different lipoprotein subclasses are separated by FPLC, concentrations of the serum components, including lipoproteins, C, CE and TAG, are diluted at least for 30-fold. It is difficult to detect the lipid components in the separated fractions by the normal colorimetric method, since the detection limits for this method is around 1 mM/L.

In this study, we used FPLC conjunction with a Superdex 200 30/100 GL SEC column to separate different serum lipoproteins, and lipid measurements in isolated fractions were completed by two sensitive fluorescence methods.

2. Materials and methods

2.1. Reagents

Cholesterol, cholesterol oleate, Amplex red and horseradish peroxidase (horseradish roots) were purchased from Sigma–Aldrich. Cholesterol oxidase (Microorganism) and cholesterol esterase (*Pseudomonas* sp.) were bought from Asahi Kasei Pharma Corporation. Nile red was purchased from invitrogen. Gel Filtration High Molecular Weight Calibration kit was purchased from GE Healthcare Life Sciences. Apolipoprotein AI/Apolipoprotein B kit was bought from BioSino Bio-technology and Science Inc in China. Apolipoprotein CIII kit was purchased from Sekisui Medical Technology in Japan.

2.2. Animals and ethical considerations

A total of 5 C57BL/6 male mice were obtained and housed in a constant temperature (23 ± 1°C) and humidity (50 ± 5%) specific pathogen free animal keeping environment. They were allowed free access to chow diet and water, and acclimatized to the environment for one week. All procedures were approved by Animal Care and Use Committee in Institute of Materia Medica.

Five healthy volunteers (blood tests showed to be non-reactive for HbsAG, anti-HCV, anti-HBc, and negative for anti-HIV 1 and 2) were enrolled. All volunteers provided signed informed consent after all the experiment procedures had been explained to them. The study protocol conformed to accepted international standards and followed the Declaration of Helsinki as revised in 2004. The protocol for the acquisition of serum samples from volunteers was approved by Ethics Committee in the Institute of Materia Medica.

2.3. Blood, serum, FPLC samples and elution buffer preparation

In the C57BL/6 mice, before 500 μL of blood sample was collected from the angular vein, all the mice were fasted for 12 h, and the whole operation was accomplished under anesthetic state by ether. For the volunteers, 1 mL of blood sample was collected from the antebrachial vein. After standing for 1 h, serum was separated by centrifugation at $6000 \times g$, and stored at −80 °C until FPLC sample preparation.

FPLC Samples Preparation: the cells were removed from the serum samples by centrifugation at $10{,}000 \times g$ for 10 min, then the supernatant was filtrated through a 0.22 μm low protein binding syringe filter (Millipore Corporation, Ireland). Gel Filtration High Molecular Weight Calibration kit FPLC samples were prepared as the serum FPLC samples.

Elution buffer was prepared as follows: dissolved 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 1.861 g Na_2EDTA, 0.2 g NaN_3 (to prevent bacterial growth in the columns) in double distilled water, adjusted the pH to 7.4 and brought the volume to 1 L. Elution buffer was filtered through a 0.22 μm filter and degassed prior to use.

2.4. Comparison of serum lipoprotein separation effect by using Superose 6 10/300GL and Superdex 200 10/300GL column

Serum lipoprotein separation was carried out on an AKATA Purifier 10 System (GE Healthcare Life Sciences) and the gel filtration chromatography was run on a single Superose 6 10/300GL (GE Healthcare, Uppsala, Sweden) or Superdex 200 10/300GL column (GE Healthcare, Uppsala, Sweden).

Two identical FPLC samples were prepared and run on a Superose 6 10/300GL and a Superdex 200 10/300GL column, respectively. After injection of 50 μL sample, the system was run with a constant flow of 0.3 mL/min. During the separation process AKATA Purifier 10 System kept a 4 °C constant temperature and all the elutions were monitored at a protein absorbance of 280 nm.

2.5. Standard protein retention volume determination on Superdex 200 10/300GL column

Separation of Blue Dextran and 5 standard proteins (thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa)) in Gel Filtration High Molecular Weight Calibration kit were carried out on an AKATA Purifier 10 System, which was in conjunction with a single Superdex 200 10/300GL column.

2.6. Serum lipoprotein separation on Superdex 200 10/300GL column and apolipoprotein analysis

Serum lipoprotein separation was carried out on a single Superdex 200 10/300GL column as described in Section 2.4. After injection of 50 μL sample the system was run with a constant flow of 0.3 mL/min, and fractions were started after 4 mL with 0.5 mL per fraction. The Apolipoprotein AI (Apo AI), Apolipoprotein B (Apo B) and Apolipoprotein CIII (Apo CIII) concentrations of each protein-containing fraction were measured by human Apo AI/Apo B kit and Apo CIII kit, respectively. Fractions containing lipoproteins were used for further analysis of total cholesterol (TC), C and TAG levels.

2.7. Lipid standards preparations and TC, C, TAG detection

C and cholesterol oleate standard mixture were diluted with 2-propanol containing 10% Triton X-100 to get a serious of gradient work solution. TAG (1.15 mM/L) standard was diluted with elution buffer to a serious of gradient work solution.

The composition and concentration of the reagent solution were given in Table 1 (Mizoguchi et al., 2004; Amundson and Zhou, 1999). In the TC assay, CE was hydrolyzed by cholesterol esterase into choleslterol, which was then oxidized by cholesterol oxidase to yield H_2O_2. In the presence of horseradish peroxidase, Amplex red reagent reacted with H_2O_2 and produced highly fluorescent resorufin. In the C assay, since cholesterol esterase was absent from the reaction, only C was oxidized by cholesterol oxidase and yielded H_2O_2, which was then detected by Amplex red in the presence of horseradish peroxidase.

Measurements of TC and C were performed in the 96-well dark micro-plate. TC and C assay were performed by adding 50 μL measurement reagent to 50 μL standard or FPLC collected sample, and allowed to react at 37 °C for 30 min. The fluorescence intensities were detected by a multi-well plate reader (Spectra Max Gemini XS, MD, American) equipped with a filter set for excitation and emission at 571 and 585 nm.