学　位　論　文

Analytical approach for the measurement of oxidized lipids and medium-chain fatty acids: Emerging lipids in human health and nutrition

(酸化脂質と中鎖脂肪酸の測定のための分析的アプローチ:

ヒトの健康と栄養に関する新規脂質)

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Abstract

Lipids and lipoproteins play a key role in the pathogenesis of coronary heart disease (CHD). Despite the strong association of oxidized lipoprotein with CHD, there is a paucity of evidence that details the chemistry and existence of molecular species of oxidized lipids in human plasma and native lipoproteins. Identification and quantification of oxidized lipids may provide valuable information to understand the process of atherogenesis and aid in CHD risk stratification. Therefore, this study aimed to scrutinize cholesteryl ester hydroperoxides (CEOOH) that can exist in plasma and unmodified lipoproteins. Furthermore, the role of triglyceride, which is predominantly transported by the triglyceride-rich lipoproteins (TRL), in the progression of atherosclerosis is uncertain. However, identification of oxidized triglycerides and their possible association with atherosclerosis may provide new insight to understand the pathogenicity of TRL. Therefore, this study is also focused on the qualitative and quantitative analysis of triglyceride hydroperoxides (TGOOH). Moreover, a condition known as triglyceride deposit cardiomyovasculopathy (TGCV), characterized by massive accumulation of triglycerides in the coronary atherosclerotic lesions, has improved clinical outcomes after dietary therapy of medium-chain triglycerides (MCT); therefore, this study also aimed to develop a quantitative assay for the measurement of capric acid (FA10:0), a medium-chain fatty acid, which may be useful in the therapeutic monitoring of such therapy.

For the analysis of CEOOH, fasting plasma was collected from six healthy volunteers. Very-low density lipoprotein (VLDL) and intermediate-density lipoprotein (IDL) were isolated from the plasma by sequential ultracentrifugation. The lipid extract of the plasma and lipoproteins were subjected for the determination of CEOOH using liquid chromatography/LTQ ion trap mass spectrometry (LC/LTQ Orbitrap). A total of six molecular species of CEOOH, namely Ch18:1-OOH, Ch18:2-OOH, Ch18:3-OOH, Ch20:4-OOH, Ch20:5-OOH, and Ch22:6-OOH, were identified on the basis of their mass spectra and retention time on the LC. Of them, Ch18:2-OOH, Ch20:4-OOH, Ch20:5-OOH, and Ch22:6-OOH were detected in all IDL samples, while only Ch22:6-OOH was detected in all VLDL samples. Except for Ch18:3-OOH, all CEOOH species were also detected in the plasma, with constant detection of Ch20:5-OOH, and Ch22:6-OOH in all plasma samples.

Similarly, the analysis of TGOOH was performed in the fasting plasma of nine human volunteers. VLDL and IDL were separated from the plasma (n=6) by sequential ultracentrifugation, followed by the isolation of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) using size-exclusion high-performance liquid chromatography (HPLC). LC/LTQ Orbitrap analysis of the lipid extract revealed 11 molecular species of TGOOH in either plasma or VLDL and IDL, of which TGOOH-18:1/18:2/16:0, TGOOH-18:1/18:1/16:0, TGOOH-16:0/18:2/16:0, TGOOH-18:1/18:1/18:1, and TGOOH-16:0/20:4/16:0 were most dominant. TGOOH-18:1/18:1/16:0 and TGOOH-16:0/18:2/16:0 were present in all plasma. Mean concentration of plasma TGOOH was 56.1±25.6 μmol per mol of triglycerides. These TGOOH molecules were carried by VLDL and IDL but not by LDL and HDL. Although IDL is relatively poor in triglycerides, the mean concentration of TGOOH in it was higher than in VLDL (512.5±173.2 vs 349.8±253.6 μmol/mol triglycerides).

Lastly, the quantitative analysis of FA10:0 was performed in blood samples taken from healthy Japanese volunteers who were in fasting (n=5, male/female=3/2, age 31±9.3 years old) and non-fasting (n=106, male/female=44/62, age 21.9±3.2 years old) states, using HPLC after derivatization with 2-nitrophenylhydrazine. This assay is analytically simple, rapid, and sensitive that specifically measures FA10:0. The inter- and intra-assay coefficient of variation of FA10:0 assay ranged from 1.7–3.9% and 1.3–5.4%, respectively, with an analytical recovery of 95.2–104.0%. FA10:0 was not detected in the fasting plasma samples. Of the plasma collected during the non-fasting state, 50 samples (47%) lacked detectable amounts of FA10:0, while the remaining samples had negligible amounts of FA10:0, with the mean of 0.3 μmol/L (SD–0.4, Max–1.6).

In conclusion, this study revealed that human plasma contains several molecular species of CEOOH and TGOOH. VLDL and IDL carry these oxidized lipids in the plasma. The existence of CEOOH and TGOOH in the TRL is possibly associated with its atherogenicity. The clinical utility of measuring CEOOH and TGOOH in these lipoproteins needs to be investigated for risk assessment of cardiovascular disease. Additionally, the human plasma contains trace amounts of FA10:0 during a non-fasting state, which may reflect dietary MCFA that have escaped the hepatic utilization. This assay can be used for monitoring the concentration of FA10:0 during dietary therapy with MCT.

Abbreviation

ATGL	: adipose tissue triglyceride lipase
CE	: cholesteryl esters
CEOOH	: cholesteryl ester hydroperoxides
CHD	: coronary heart disease
CVD	: cardiovascular diseases
EIC	: extracted ion chromatograms
ESI	: electrospray ionization
FA	: fatty acids
FAOOH	: fatty acid hydroperoxides
GC	: gas chromatography
HDL	: high-density lipoprotein
HDL-C	: HDL-cholesterol
HPLC	: high-performance liquid chromatography
IDL	: intermediate-density lipoprotein
LCFA	: long-chain fatty acids
LC/MS	: liquid chromatography/mass spectrometer
LDL	: low-density lipoprotein
LDL-C	: LDL-cholesterol
LOOH	: lipid hydroperoxides
MCFA	: medium-chain fatty acids
MCT	: medium-chain triglycerides
MDA	: malondialdehydes
MetS	: metabolic syndrome
NCEP	: National Cholesterol Education Program
nLDL	: native LDL
NPH	: 2-nitrophenylhydrazine
oxLDL	: oxidized LDL
PAGE	: polyacrylamide gel electrophoresis
PCOOH	: phosphatidyl choline hydroperoxides

PUFA : polyunsaturated fatty acids

ROS : reactive oxygen species

RT : retention time

SDS : sodium dodecylsulphate

SRM : selective reaction monitoring

TGCV : triglyceride deposit cardiomyovasculopathy

TGOOH : triglyceride hydroperoxides

TIC : total ion chromatogram

TRL : triglyceride-rich lipoproteins

VLDL : very low-density lipoprotein

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Chapter 1: Preface

1.1. General Introduction

Lipids are ubiquitous in the human body, playing diverse physiological functions. Most importantly, it forms a major structural component of biological membranes, acts as an additional source of metabolic fuel and energy storage, insulates neurons, serves as chemical messengers and mediates signal transduction. However, unfortunately, lipids are equally associated with a number of diseases, some of which remain as a global health problem in today's clinical practice. For instance, cardiovascular disease (CVD), and lipid storage disorders including obesity and metabolic syndrome (MetS) are increasing at an alarming rate and are major threats to human health from the past few decades. Oxidative modification and excessive storage of lipids are two important arenas in the modern lipidology that have dramatic influences on human health and nutrition. As a consequence, much attention has been focused on certain lipids in order to – elucidate its association in the pathogenesis of diseases, identify potential biomarkers for the prediction of future risks, and formulate effective management for the minimization of complications. On the other hand, the interest of using certain lipids as a dietary intervention to benefit various clinical conditions is also increasing. Polyunsaturated fatty acids (PUFA) are known to be protective against CVD, and the recent studies are revealing potential clinical utility of medium-chain fatty acids (MCFA).

1.1.1. Lipids and Coronary Heart Disease

Lipids, being relatively hydrophobic, typically circulate in the form of lipoproteins. Unique structural conformation of the lipoprotein, with a core of hydrophobic

nonpolar lipids – triglycerides and cholesteryl esters (CE) surrounded by relatively polar or amphipathic lipids – phospholipids and free cholesterol along with one or more specific proteins (apolipoproteins), permits transportation of lipids to meet their metabolic functions. Apart from structural component, the apolipoproteins also serve as coenzymes, activating several enzymes involved in the metabolic pathway of lipoprotein metabolism, and act as recognizing units for certain cell surface receptors. Based on density as determined by ultracentrifugation, lipoproteins are broadly categorized into – chylomicrons, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL), each with discrete biological properties and physiological functions. Because of the strong association of lipids and lipoproteins in CVD, especially coronary heart disease (CHD) (also known as coronary artery disease), laboratory measurements of lipoproteins and their constituents play a crucial role in the clinical practice and research investigations.

CHD, an atherosclerotic disease of coronary artery, is characterized by narrowing of the artery lumen due to deposition of lipid-rich plaque that can trigger the inflammatory response and ultimately thrombotic events. Several hypotheses have been postulated to account the pathogenesis of atherosclerosis. The "response-to-injury" hypothesis of Ross is probably the most widely accepted one.[1] It is focused on the smooth muscle proliferation in the lesions of atherosclerosis in response to injury to the endothelium and emphasized the role of platelets in this phenomenon. Several risk factors, including hyperlipidemia were identified that can contribute to the endothelial injury.[2] Emergence of the "lipid oxidation" hypothesis of Steinberg and colleagues provided another insight in understanding the vascular injury. It illustrated the role of macrophage-derived foam cells in the early atherogenesis.[3] The existence of "scavenger receptor" allows the unregulated uptake of cholesterol in the form of

oxidized LDL (oxLDL) that further promotes the formation of foam cells.[4] In general, the process of atherosclerosis, though progresses slowly, is a complex phenomenon involving multiple factors – inflammation, oxidative stress, lipid accumulation and thrombotic events.

1.1.2. Cardiac Risk Factors

Identification of individuals who currently lack symptoms but are at high risk for the development of future coronary events is a critical issue in the primary prevention of CVD because appropriate early intervention can prevent or delay the progression of the atherosclerotic disease. Laboratory medicine plays a key role in the risk stratification of future CHD, with serum lipids and lipoproteins being routinely used for this purpose. Research from experimental animals, laboratory investigation, epidemiological and genetic studies indicate that serum cholesterol, particularly carried in LDL is the most promising risk factor for CHD. National Cholesterol Education Program (NCEP) ATP III emphasized LDL-cholesterol (LDL-C) as a major cause of CHD and considered it as the primary target of therapy.[5] HDL-cholesterol (HDL-C), on the other hand, protects against the development of atherosclerosis and is inversely correlated with the risk of CHD. Serum triglyceride is considered as an independent cardiovascular risk factor. VLDL is major triglyceride-rich lipoproteins (TRL) in the fasting state, which is primarily responsible for the transportation of endogenous fats. However, VLDL also comprises 10 – 15% of serum total cholesterol. The percentage content of cholesterol in VLDL increases during the course of metabolism and get transformed into VLDL remnants, then subsequently to intermediate-density lipoproteins (IDL). Growing evidence indicates that both VLDL and IDL may play an important role in the atherogenesis. Apart from

these lipids and lipoproteins, several potentially modifiable risk factors have also been identified and formed the rationale for intervention, that include – diabetes, hypertension, obesity and MetS, smoking status, and thrombogenic/ hemostatic state.[5]

Despite the great importance of these traditional risk factors for the risk stratification of CHD, number of studies have questioned on their adequacy to predict the future risks. For instance, almost half of all coronary events occur among the individuals without dyslipidemia.[6] Furthermore, significant numbers of coronary events occurred in the absence of any major classical risk factors, including hyperlipidemia, hypertension, diabetes and smoking.[7] Therefore, in the recent years, intensive researches are focused to identify novel risk factors that can enhance the predictability of future CHD. A number of "emerging risk factors" has been identified with varying degree of clinical utility. Some of the promising emerging risk factors include – high-sensitivity C-reactive protein (hsCRP), lipoprotein (a), homocysteine, small dense LDL, apolipoprotein B and A-1, lipoprotein remnants, LDL particles, and oxidized LDL. Recently, we reported predictive value of product of serum calcium and phosphorus as a cardiac risk factor.[8]

1.1.3. Oxidized Lipoproteins

Oxidation of lipoproteins is another key factor that can elicit the atherogenesis.[9,10] Therefore, oxidized lipoproteins have received great attention, both to understand the pathophysiology of atherosclerosis and prediction of the risk. Back in 1983, Brown and Goldstein (Nobel prize – 1985) reported that the structural modification of circulating LDL is proatherogenic.[4] There is a direct involvement of oxLDL in the atherosclerosis. The oxLDL are unrecognizable by native LDL receptors and largely taken up by macrophages through the scavenger receptors in an unregulated fashion

to form foam cells. Additional proatherogenic properties of oxLDL include chemo-attraction of circulating monocytes, promotion of the monocytes differentiation to the tissue macrophage, and inhibition of the motility of resident macrophages.[11] It is now well evident that LDL oxidation can occur *in vivo* and oxLDL have been identified in human atherosclerotic lesions, further supporting the involvement of oxLDL in CHD. Thus, it appears that direct measurement of oxLDL could serve as an early marker for atherosclerosis and is targeted for therapeutic intervention. Considerable numbers of immunoassay specific for various epitopes of oxLDL are currently available. Recently, Sakurai et al. reported a novel monoclonal antibody that can react with small dense oxLDL and triglyceride-rich oxLDL.[12,13] Furthermore, circulating anti-oxLDL antibodies have also been detected in the serum, with titers correlating with the progression of atherosclerosis. We previously demonstrated that anti-oxLDL antibody is associated with hypertension, diabetes and MetS and its level correlates with major cardiac risk factors.[14,15] Several other approaches, including carbon nanotube sensor, have been applied for the detection of oxLDL.[16,17] However, the clinical relevance of measurement of oxLDL is yet to be established; hence, are not in routine use.

Although oxLDL are primarily blamed for atherosclerosis, a growing body of evidence is revealing the potentiality of VLDL and IDL to initiate the process of atherosclerosis; therefore, the role of oxidized form of these TRL cannot be negated.[18-21] Both VLDL and IDL have been recovered from human aorta and atherosclerotic plaques.[22-24] Furthermore, oxidized TRL are considered as proatherogenic and can increase cellular CE in macrophages, resulting in formation of foam cells.[25]

1.1.4. Oxidized Lipids

A large number of oxidized forms of lipids (cholesterol, triglycerides, phospholipids, glycolipids and fatty acids) have been documented *in vitro* and some of them are also identified *in vivo*. Presence of oxidized lipids has been well documented in human atherosclerotic lesions.[26,27] Therefore, study of oxidized lipids may provide valuable information about the atherogenesis. For instance, cholesteryl ester hydroperoxides (CEOOH) are the major biologically active components of oxLDL, which in turn stimulate a wide variety of cellular and molecular processes involved in atherosclerosis.[28]

The precise mechanism of the lipid peroxidation *in vivo* is largely unknown. Fatty acids (FA), particularly polyunsaturated fatty acids (PUFA), are vulnerable for the peroxidation. It is generally believed that various reactive oxygen species (ROS) generated in our body, either during normal metabolism or due to environmental factors, are responsible for oxidative modification of biomolecules.[29] Lipid peroxidation is usually initiated by the abstraction of a hydrogen atom from a carbon adjacent to double bonds, resulting in the formation of conjugated dienes. Subsequently, oxygen molecule is incorporated into it, leading to the formation of lipid hydroperoxides (LOOH). Therefore, LOOH is considered as an early product indicator of lipid peroxidation. LOOH can further degrade to the final products – malondialdehyde (MDA) and 4-hydroxynonenal. Furthermore, LOOH can generate other highly reactive and oxidizing radicals like lipid-peroxyl (LOO$^{\bullet}$), oxyl (LO$^{\bullet}$), and epoxy-allylic peroxyl (OLOO$^{\bullet}$). All of these radicals, in turn, can lead to peroxidation of adjacent lipids; therefore, initiating the chain peroxidation reaction.[30] Moreover, LOOH may also oxidize apolipoproteins, making it unrecognizable by its receptors

and disrupting its metabolic function.[31] Therefore, oxidized lipid in the lipoprotein is one of the key promoting factors for its atherogenicity.

Since LOOH are the major reaction products of lipid oxidation, detection of LOOH in plasma or lipoprotein fraction is an indicator of the oxidative change.[32] Despite its importance in the atherosclerotic process, the qualitative and quantitative evaluation of LOOH in the biological samples have been largely limited, possibly due to structural variability and instability, rapid clearance from circulation, too low concentration for easy detection, and the lack of appropriate internal and external standards. Thiobarbituric acid reactive substances (TBARS) assay is perhaps the most frequently used markers as an indirect reflection of lipid peroxidation. However, it has low specificity, as it detects a wide group of aldehydes and alcohols present in the sample. Similarly, monitoring the formation of conjugated dienes by measuring optical absorbance can provide information about the oxidative change in the lipids but it is largely limited to monitor oxidative change during artificial oxidation of lipids. Likewise, the colorimetric method by ferrous oxidation using xylenol orange,[33] and iodometric method[34] have been used for detection of LOOH and reported measurable amount of lipid peroxides in native LDL. However, results from more specific method – high-performance liquid chromatography (HPLC) indicates that human plasma from healthy individuals and native LDL (nLDL) are free from detectable amounts of LOOH.[35] HPLC with post-column detection based on fluorometry,[36,37] chemiluminometry[38-40] and electrochemistry[41,42] have been proposed for the measurement of CEOOH, phosphatidyl choline hydroperoxides (PCOOH), and triglyceride hydroperoxides (TGOOH). Using HPLC, Hui et al. found that normal young human plasma contain 189±87 nM of CEOOH but no TGOOH.[43] On the other hand, the advanced liver disease is associated with significant elevation of both

CEOOH and TGOOH.[43] PCOOH has been demonstrated in human plasma ranging from 0.01 to 0.5 μM using chemiluminescence HPLC. PCOOH concentration is significantly elevated in diseased condition like diabetes, and is mainly carried by LDL and VLDL.[44]

Utilization of liquid chromatography/mass spectrometer (LC/MS) has revolutionized the study of oxidized lipids because it is targeted to specific molecular species. Hydroperoxides of CE, phospholipids and triglycerides have been identified using LC/MS.[45-47] Hui et al. detected several molecular species of CEOOH and TGOOH in artificially-oxidized LDL and HDL using highly sensitive reversed-phase liquid chromatography with a hybrid linear ion trap-Orbitrap mass spectrometer.[45,47] Interestingly, both CEOOH and TGOOH were detected in oxLDL but not in nLDL.[45,47,48]

1.1.5. Serum Triglycerides and Oxidized Triglycerides

The majority of the plasma triglycerides are carried in TRL with density <1.019 kg/L that includes chylomicrons, VLDL and IDL. Initially, National Institutes of Health (NIH) consensus conference on "triglyceride and CHD" failed to establish fasting triglycerides concentration as an independent risk factors.[49] After that, a large number of epidemiological, clinical and experimental studies, continue to support plasma triglycerides as an independent risk factor for CHD.[50-55] The results of study by Asia Pacific Cohort Studies Collaboration demonstrated that serum triglyceride level is an independent determinant of cardiovascular risk across a broad population group within the Asia-Pacific region.[56] NCEP identified serum triglycerides as a marker of atherogenic remnant lipoproteins and other lipids risk factors (small LDL particles and low HDL).[5] Serum triglyceride is also correlated with non-lipid factors – obesity,

hypertension, diabetes, and cigarette smoking.[57] In the recent years, much focus has been given to postprandial (non-fasting) triglycerides as a better marker for the cardiac risk prediction.[58-60] However, there is no consensus on whether triglycerides itself is causative of CHD, or it is just a marker of remnant lipoproteins or the metabolic changes that it brings, for example the reduction of HDL, is atherogenic. Moreover, fatty acids in the triglycerides are equally vulnerable to peroxidation. However, less importance is given to the measurement of oxidized triglycerides. The influence of the oxidized triglycerides in the atherogenesis is yet to be explored. In general, the specific role of triglycerides in the progression of CHD is uncertain and has long been controversial.[49,61,62]

1.1.6. Triglyceride Storage Disorders

Excessive deposition of triglycerides, in both native (adipose tissue) and ectopic site, is another important arena that needs serious attention. The prevalence of obesity is increasing worldwide at an alarming rate and even considered as a pandemic.[63] The etiology of obesity is multifactorial. Although our understanding of the role of genetics is increasing, obesity is basically caused by the chronic energy surplus in which dietary intake of calories exceeds the energy expenditure resulting in net accumulation of triglycerides. Obesity plays pivotal role in the pathogenesis of metabolic and CVD; therefore, considered as an important risk factor for CHD.[64] The risk of CHD is particularly raised when abdominal obesity, a component of MetS as defined by NCEP ATP III, is present.[5] Furthermore, obesity and overweight are often associated with atherogenic dyslipidemia including low HDL and high LDL, and high VLDL.[65] On the other hand, obesity is characterized by remarkable increase in non-esterified (free) FA and triglycerides in the circulation. As consequences, triglycerides

are excessively deposited in the ectopic sites, including the liver, skeletal muscle, heart, kidney and pancreatic β-cells. Less commonly, such ectopic lipid storage disorders are resulted due to genetic diseases and inborn errors of metabolism.

1.1.7. Triglyceride Deposit Cardiomyovasculopathy

In 2008, Hirano et al. from Osaka University reported a new disease entitled "triglyceride deposit cardiomyovasculopathy (TGCV)," in The New England Journal of Medicine.[66] The stunning feature of this disease is that, it is triglycerides but not the cholesterol that was accumulated in the atherosclerotic lesion. Although the heart largely depends on triglycerides to meet its energy demand, lipid droplets rarely accumulate in the cardiac muscle under normal circumstance. However, this disease, sometimes referred as "obesity of the heart," is characterized by the massive accumulation of triglycerides in the coronary atherosclerotic lesions and the myocardium despite of normal serum triglyceride concentration.[67]

The molecular mechanism behind TGCV is mainly due to mutation in the adipose tissue triglyceride lipase (ATGL, also known as PNPLA2) or its activator – comparative gene identification-58 (CGI-58) but a few are idiopathic. ATGL is responsible for the intracellular hydrolysis of depot triglycerides to liberate FA as energy substrate. Though ATGL is primarily expressed in adipose tissue, its deficiency can result excessive deposition of triglycerides not only limited to adipose tissue but also in various ectopic sites including the muscle.[68,69] Conversely, the overexpression of myocardial ATGL can reduce triglyceride deposition in the heart.[70] A recent study revealed that peroxisome proliferated activator receptors (PPARPs) and related genes are up-regulated in myocardium of TGCV patients, leading to increase in uptake of long-chain fatty acids (LCFA) and its storage as neutral fat.[71]

Interestingly, it appears that MCFA incorporate into the cellular lipids at a lower rate than LCFA and induce a lower accumulation of triglycerides in the ATGL-mutated cells.[72] In addition, the cytoplasmic triglycerides are degraded through two separate pathways, one being specific to long-chain triglycerides (i.e., ATGL-mediated) and the others one specific to medium-chain triglycerides (MCT). Therefore, *in situ* degradation of MCFA containing triglycerides is not defective in ATGL-mutated cells, which form rationale to use dietary intervention with MCT for the management of TGCV.

1.1.8. Medium-Chain Triglycerides and Medium-Chain Fatty Acids

Medium-chain triglycerides (MCT) are ester of MCFA mainly – octanoic acids (FA8:0) and capric acid (FA10:0) with glycerol. Naturally, coconut and palm kernel oils are rich in MCT with more than 50 wt% of MCFA. MCT is also found in the milk, where MCFA comprises 4-10% of all fatty acids.[73] Therefore, dairy products are the important dietary source of MCFA in human nutrition.

Several distinguishing features make MCT metabolism unique to that of other LCFA containing oils and fats. First, MCT is rapidly hydrolyzed by gastric lipase even in the absence of bile and are readily absorbed into the enterocytes. Therefore, MCT has been used in the treatment of pancreatic insufficiency and fat malabsorption syndrome from decades. Second, unlike lymphatic transportation of LCFA through chylomicrons, dietary MCFA are directly carried to the liver through the portal circulation. Then MCFA are completely metabolized within the hepatocytes.[74] Therefore, plasma concentration of MCFA is negligible during ordinary diet. Third, the entry of MCFA into mitochondria bypasses the rate-limiting carnitine transport system, resulting in its rapid and unregulated utilization. Fourth, as mentioned earlier,

the cellular utilization of MCT is independent to the rate-limiting action of ATGL.[72]

The interest of dietary intervention with MCT is increasing to benefit various clinical conditions. MCT has been reported to reduce body weight and prevent obesity,[75-77] liver diseases,[78,79] MetS[80] and insulin resistance.[81,82] Although the mechanism of action of MCT in cardiac metabolism remains largely unknown, interest of using MCT to benefit the patients with cardiac diseases, has increased in the recent years.[83] Due to the unique metabolic properties of MCT, it appears that MCFA, particularly FA10:0 may improve the clinical outcome in TGCV patients. Currently, the Japan TGCV study group is involved in extensive research to assess the potential utility of using MCT as a drug for the treatment of TGCV.

1.2. Rationale of Study

Identification and quantification of LOOH may provide valuable information in understanding atherogenesis and aid in the risk stratification of CHD. In earlier studies, the identification of LOOH was mainly focused on artificially-oxidized lipoproteins or plasma. Hui et al. observed several molecular species of CEOOH and TGOOH in the artificially-oxidized LDL and HDL.[45,47] Despite being rich in cholesterol, CEOOH was not detected in both native LDL and HDL, however, normal human plasma contains detectable levels of some molecular species of CEOOH, [45,48] which prompted us to investigate whether the CEOOH species detected in the plasma were carried in VLDL and/or IDL. Moreover, though relatively rich in triglycerides, both VLDL and IDL are potentially proatherogenic; oxidized triglycerides in these TRL may increase their atherogenicity. However, TGOOH is undetected in plasma and lipoproteins using conventional assays. Therefore, this study focuses on identification and quantification of TGOOH in the TRL and plasma using highly

sensitive reversed-phase lipid chromatography with a hybrid linear ion trap mass spectrometer (LC/LTQ Orbitrap). Furthermore, the quantitative analysis of FA10:0 in the biological samples, and its association with clinical signs and symptoms in patients receiving MCT dietary therapy, is key to understanding the mechanism underlying the benefits of such dietary therapy; however, the measurement of FA10:0 in the plasma remains challenging due to its trace availability in the systemic circulation and volatility, compared to LCFA. Therefore, it became necessary to develop a highly sensitive, reliable assay that can specifically measure FA10:0.

1.3. Aims of Study

General objective

- To analyze CEOOH, TGOOH, and MCFA in human plasma.

Specific objectives

- To develop analytical methods for the detection of CEOOH and TGOOH in the plasma and TRL.
- To detect and identify the molecular species of CEOOH in the plasma and TRL.
- To identify the molecular species of TGOOH in the plasma and its distribution among the lipoprotein.
- To find the relative concentration of TGOOH in the healthy human plasma and TRL.
- To develop a simple, reliable and highly sensitive method for the determination of FA10:0 in biological samples.
- To find the normal plasma concentration of FA10:0 in healthy volunteer during fasting and non-fasting state.

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Chapter 2: Identification of Molecular Species of Cholesteryl Ester Hydroperoxides in Very Low-Density and Intermediate-Density Lipoproteins

2.1. Introduction

It is widely accepted that the oxidation of lipoproteins has immense effect on the development of atherosclerosis. Since LOOH are the major reaction products of lipoprotein oxidation, its detection in lipoprotein fractions can indicate the oxidative change in the lipoprotein and thus may be associated with atherosclerosis. Though oxLDL are primarily blamed for atherosclerosis, the role of VLDL and IDL in their oxidized forms cannot be neglected.[1-5]

Accumulation of CEOOH has been well documented in human atherosclerotic lesions.[6,7] Further, CEOOH are the major biologically active components of minimally oxidized LDL, which, in turn, stimulate a wide variety of cellular and molecular processes involved in atherosclerosis.[8] Moreover, copper-mediated oxidation can induce increase formation of CEOOH in LDL as well as HDL.[9] Thus, CEOOH can be considered as one of the most relevant lipids in atherosclerosis.

Despite the importance of CEOOH in atherosclerotic process, the qualitative and quantitative evaluation of CEOOH in lipoproteins has been largely limited, possibly due to structural variability and instability, rapid clearance from circulation, and too low concentration for easy detection, and the lack of standard CEOOH.

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Therefore, there is a paucity of evidence that details the chemistry and existence of CEOOH in native plasma lipoproteins, most of all, in TRL.

Several methods have been reported for the measurement of LOOH in human plasma and specific lipoprotein fractions, namely colorimetric method by ferrous oxidation using xylenol orange,[10] iodometric measurement,[11] HPLC with post-column detection based on fluorometry,[12,13] chemiluminometry[14-16] and electrochemistry.[17,18] In the recent years, LC/MS has became a powerful tool in the study of LOOH. LC/MS methods for detection of CEOOH,[9] PCOOH,[19] and TGOOH[20] have been reported. Hui et al. previously identified several CEOOH species in chemically-oxidized lipoprotein fractions using highly sensitive reversed-phase liquid chromatography with a hybrid linear ion trap-Orbitrap mass spectrometer (LC/LTQ XL Orbitrap, Thermo Fisher Scientific, Waltham, MA, USA) and in-house-built standard compounds.[9] In the previous study, CEOOH were not detected in the isolated LDL and HDL fractions in their native forms while detected in the plasma.[9] This finding led us to a question whether the CEOOH species detected in plasma were carried in VLDL and/or IDL. Thus, this study was targeted to detect and identify CEOOH in TRL.

2.2. Materials and Methods

2.2.1. Chemicals

Cholesteryl oleate monohydroperoxide (Ch18:1-OOH), cholesteryl linoleate monohydroperoxide (Ch18:2-OOH), and cholesteryl linolenate monohydroperoxide (Ch18:3-OOH) were used as standards, which were synthesized chemically in our laboratory reported elsewhere.[21] All other chemicals and solvents were of analytical

grade and obtained from Wako Pure Chemical Industry (Osaka, Japan), unless specified.

2.2.2. Plasma Preparation

A fasting EDTA blood (10 mL) was collected from six apparently healthy volunteers (Range, 27 – 36 years.). Written informed consent was obtained from all human volunteers. The samples were immediately kept on ice and centrifuged at 2000 × g for 10 min at 4°C, within 30 min. A portion of the plasma (1.0 mL) was immediately stored at -80°C until analyzed by LC/LTQ Orbitrap, while remaining were used for isolation of VLDL and IDL. Additional plasma sample was collected from three of the six donors and analyzed freshly by the LC/LTQ Orbitrap to rule out possible auto-oxidation during storage.

2.2.3. VLDL and IDL Isolation

Sequential ultracentrifugation was used to isolate VLDL and IDL from the plasma.[22] Briefly, ultracentrifugation was performed using a near-vertical tube rotor (MLN-80, Beckman Coulter, Fullerton, CA, USA) on a model Optima MAX (Beckman Coulter). Plasma (2.0 mL) mixed with 6.0 mL of d=1.006 kg/L solution containing NaCl (1.14%, w/v), EDTA-2Na (0.01%, w/v) and 1M NaOH (0.1%, v/v) was centrifuged at 50,000 rpm for 14 hours at 4°C, followed by collection of upper fraction (2.5 mL) as VLDL. The remaining fraction was then adjusted to d=1.019 kg/L with KBr solution and centrifuged at 40,000 rpm for 20 hours at 4°C. Upper fraction (2.5 mL) containing IDL was isolated. The isolated VLDL and IDL were concentrated by ultrafiltration using XM-50 and Millipore Amicon Bioseparations Stirred Cells (Thermo Fisher Scientific, Waltham, MA, USA).

2.2.4. Assessing the Purity of VLDL and IDL

The purity of isolated VLDL and IDL was checked by determining their chemical composition, apolipoprotein study, and characteristic motility in polyacrylamide gel disc electrophoresis (LipoPhor, Jokoh Co, Tokyo, Japan) and agarose gel electrophoresis using universal electrophoresis film (Helena Laboratory, Beaumont, TX, USA).[19,23] Total cholesterol (TC), free cholesterol (FC), triglycerides and phospholipids in the VLDL and IDL were measured by automated enzymatic methods (Kyowa Medex Co, Ltd, Tokyo, Japan). CE concentration was calculated by multiplying the esterified cholesterol concentrations (obtained by subtracting FC from TC) by 1.72.[24] Apolipoproteins study was done in 3-10% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (ATTO Bioscience and biotechnology, Tokyo, Japan) after reduction with 2-mercaptoethanol and stained with SimpleBlue™ SafeStain-Coomassie Brilliant Blue (Invitrogen, USA). Protein content in VLDL and IDL were measured by the modified Lowry method.[25]

2.2.5. Preparation of Sample for LC/LTQ Orbitrap

Lipids from VLDL and IDL were extracted by the previously reported method.[9,19] Briefly, 0.1 mL of the lipoprotein, each with total lipid concentration of 0.5 g/L, were mixed with 0.4 mL of freshly prepared 0.005% (w/v) 2,6-di-*tert*-butyl-*p*-cresol (as an antioxidant) in acetonitrile and 2.0 mL of chloroform. For extraction of lipid from plasma, 0.2 mL of the plasma was mixed with 0.8 mL of freshly prepared 0.005% 2,6-di-*tert*-butyl-*p*-cresol in acetonitrile and 2.0 mL of chloroform. The mixture was then vortex mixed vigorously for 30 sec and centrifuged at 3,000 × g for 10 min at 4°C. The extraction was performed twice. The chloroform layer was collected

followed by complete evaporation under vacuum and the residue was dissolved in 300 μL of methanol.

2.2.6. LC/LTQ Orbitrap

Ten μL of the lipid extract was injected onto a reversed-phase Syncronis C18 HPLC column [50 mm × 2.1 (i.d.) mm; particle size 1.7 μm] (Thermo Fisher Scientific, Waltham, MA, USA), maintained at 60°C. Gradient elution was performed with a mobile phase composed of 10 mmol/L aqueous ammonium acetate (Solvent A) and 2-propanol (Solvent B). The HPLC gradient elution program was: 0.00 – 1.00 min 50% A and 50% B; 5.01 – 7.00 min 0% A and 100% B; 7.01 - 10.0 min 50% A and 50% B at a flow rate of 0.2 mL/min.

High-resolution mass spectrometric analysis was performed using LTQ XL Orbitrap mass spectrometer combined with Surveyor MS pump and an autosampler.[9,19] Electrospray ionization (ESI) tandem mass spectrometry analysis was performed in positive-ion mode and mass spectra were obtained in Fourier-transform mode, with a target mass resolution of R=60,000 at m/z 400 under automatic gain control set to 5.0×10^5 as the target value. The ion spray voltage was set at 5.0 kV, with a scan range of m/z 150–1000. The trap fill-time was set at 500 ms. Nitrogen was used as sheath gas (set at 50 arbitrary units). CEOOH was detected as the $[M+NH_4]^+$. Extracted ion chromatograms (EIC) were drawn with the mass tolerance set at 5.0 ppm.

2.2.7. Data Processing

By use of Qual Browser 2.0 software (Thermo Fisher Scientific), each survey spectrum was converted into a peak list, which reported m/z, relative intensity (normalized to the most abundant peak), and the sum composition for each peak

detected with a signal-to-noise ratio above 6. Sum compositions were calculated from the determined precursor masses, assuming the settings: mass tolerance within ±5.0 ppm; even-electron ions. Assumed atomic compositions were restricted to: nitrogen, 1 to 2 atoms per molecule; oxygen, 2 to 15 atoms; and unrestricted carbon and hydrogen.

2.3. Results

The study of chemical composition of VLDL and IDL, apolipoprotein composition determined by SDS-PAGE and characteristic mobility of lipoprotein in LipoPhor indicate that the isolated lipoprotein fractions were pure.

2.3.1. Detection of Ch18:1-OOH in VLDL and IDL

LC/LTQ Orbitrap in positive-ion mode was used for qualitative analysis of CEOOH in the extracts of VLDL, IDL and plasma. The first step of this approach for the detection of hydroperoxides was to collect high-resolution spectra. The spectra were acquired for three synthetic CEOOH standards, and the extracts of VLDL and IDL.

Figure 2.1A-C shows EIC of *m/z* 700.6238 in positive-ion mode for synthetic Ch18:1-OOH (40 pmol), VLDL and IDL respectively; their corresponding mass spectra are shown in Figure 2.1A'-C'. For the synthetic Ch18:1-OOH, one peak was observed at a retention time (RT) of 7.07 min (Figure 2.1A); the corresponding mass spectrum obtained is shown in Figure 2.1A', showing $[M+NH_4]^+$ at *m/z* 700.6257 (elemental composition $C_{45}H_{82}O_4N$, theoretical mass 700.6238).

A total ion chromatogram (TIC) of VLDL shows absence of clearly defined HPLC peaks and large over-lapping peaks (Figure 2.1B). However, extraction of a particular signal, *m/z* 700.6238, from the TIC led to a much better defined chromatogram. One peak was observed at RT 7.00 min, and the corresponding mass

spectrum obtained is shown in Figure 2.1B'. The peak observed at *m/z* 700.6265 corresponding to $[M+NH_4]^+$, and the ion has the same elemental composition and theoretical mass as the ion from synthetic Ch18:1-OOH, thus the peak at 7.00 min in Figure 2.1B was identified as Ch18:1-OOH in VLDL. In the same way, the peak at 7.05 min in Figure 2.1C obtained from IDL was identified as Ch18:1-OOH.

2.3.2. Detection of Ch18:2-OOH in VLDL and IDL

Chromatogram and mass spectrum of Ch18:2-OOH obtained from the standard, VLDL and IDL were shown in Figure 2.2. Standard Ch18:2-OOH was eluted at the RT of 7.00 min (Figure 2.2A) and corresponding mass spectrum revealed $[M+NH_4]^+$ at *m/z* 698.6086 (elemental composition $C_{45}H_{80}O_4N$, theoretical mass 698.6082) (Figure 2.2A'). In Figure 2.2B', the base peak at *m/z* 698.6088 corresponded to $[M+NH_4]^+$ have the same elemental composition and theoretical mass as the ions from standard Ch18:2-OOH (Figure 2.2A'), implying the peak at 7.02 min in Figure 2.2B obtained from VLDL is Ch18:2-OOH. Similarly, the peak at 7.01 min in Figure 2.2C obtained from IDL was identified as Ch18:2-OOH.

2.3.3. Detection of Ch18:3-OOH in VLDL and IDL

Ch18:3-OOH was detected in VLDL and IDL fractions but not in plasma. Figure 2.3A–C shows EIC of *m/z* 696.5925 for synthetic Ch18:3-OOH (40 pmol), VLDL, and IDL; and their corresponding mass spectra are shown in Figure 2.3A'–C', respectively. Synthetic Ch18:3-OOH was eluted at the RT of 6.96 min (Figure 2.3A); the corresponding mass spectrum is shown in Figure 2.3A', elucidating $[M+NH_4]^+$ at *m/z* 696.5922 (elemental composition $C_{45}H_{78}O_4N$, theoretical mass 696.5925). In Figure 2.3B', a peak at *m/z* 696.5936 corresponding to $[M+NH_4]^+$ have the same elemental composition and theoretical mass as the ions from synthetic Ch18:3-OOH,

indicating the peak at 6.97 min in Figure 2.3B from VLDL is Ch18:3-OOH. Similarly, Ch18:3-OOH was also identified in IDL.

2.3.4. Detection of other Molecular Species of CEOOH

Cholesteryl arachidonate monohydroperoxide (Ch20:4-OOH), cholesteryl eicosapentaenate monohydroperoxide (Ch20:5-OOH), and cholesteryl docosahexaenate monohydroperoxide (Ch22:6-OOH) were also detected in VLDL, IDL and plasma by the use of LC/LTQ Orbitrap. All characteristic ions of CEOOH detected in VLDL, IDL and plasma in a sample are summarized in Table 2.1.

2.3.5. Detection of CEOOH in Plasma

Five molecular species of CEOOH were detected in plasma. All of the plasma contained Ch22:6-OOH and Ch20:5-OOH. While, Ch18:1-OOH, Ch20:4-OOH and Ch18:2-OOH were found in most of the plasma (Table 2.2). In contrast, no Ch18:3-OOH was detected in plasma samples. The TIC and spectra of a plasma extract are shown in Figure 2.4.

2.3.6. Distribution of CEOOH

This study identified six molecular CEOOH species overall, namely Ch18:1-OOH, Ch18:2-OOH, Ch18:3-OOH, Ch20:4-OOH, Ch20:5-OOH, and Ch22:6-OOH (Table 2.2). Of them, Ch18:2-OOH, Ch20:5-OOH, Ch20:4-OOH and Ch22:6-OOH were detected in all IDL samples, while only Ch20:4-OOH was detected in all VLDL samples. Except Ch18:3-OOH, all other CEOOH species were detected in all frozen plasma, with constant detection of Ch20:5-OOH and Ch22:6-OOH. The fresh samples contained Ch18:2-OOH, Ch20:4-OOH, Ch18:1-OOH, Ch20:5-OOH and Ch22:6-OOH overall, of them latter three were constantly detected. No

significant difference in distribution of CEOOH was observed between the fresh plasma samples and the stored frozen plasma samples.

2.4. Discussion

This study reported a LC/LTQ Orbitrap method that is sensitive enough to detect CEOOH in human plasma and native lipoprotein fractions using in-house-built standards. Use of this method enabled us to identify six CEOOH molecular species in VLDL and IDL, namely Ch18:1-OOH, Ch18:2-OOH, Ch18:3-OOH, Ch20:4-OOH, Ch20:5-OOH and Ch22:6-OOH, on the basis of their mass spectra. The possibility of auto-oxidation during storage was negated, since fresh plasma samples showed essentially the same results as the frozen samples.

This successful demonstration of CEOOH in VLDL and IDL is attributed to several factors. Firstly, we used three authentic standards (Ch18:1-OOH, Ch18:2-OOH and Ch18:3-OOH), which enabled us to develop an unequivocal method for the identification on the basis of their mass spectra and RT on LC. Secondly, we used LC/LTQ Orbitrap, which can obtain accurate *m/z* values for adducts of the molecular ions from individual molecules by high mass resolution. It provides high-resolution EIC within ±5.0 ppm relative mass deviation and spectra with selected extraction of ions at ±5.0 ppm accuracy. Most importantly, this method has a high analytical sensitivity of 0.1 pmol.

Although previous studies demonstrated the presence of CEOOH in healthy plasma, the molecular species of CEOOH were not specified.[12,26,27] Furthermore, lipoprotein source of the detected CEOOH was not specified in these studies. In the present report, we focused on VLDL and IDL, because the role for TRL in atherosclerosis is poorly understood. However, both VLDL and IDL have ability to

induce foam cell formation *in vitro,* and the oxidized forms of these lipoproteins have been identified in atherosclerotic lesions.[3,28,29] The detection of CEOOH in VLDL and IDL in the present study might support a possible involvement of these lipoproteins in atherogenic process.

The physiologically relevant mechanisms underlying oxidation of CE *in vivo* are largely unknown. Although VLDL carry mainly endogenous lipids, possible integration of oxidized lipids from the diet into CE during assembly of VLDL in the liver cannot be excluded.[30,31] In addition, possibly, the oxidation of CE in VLDL and IDL can occur during systemic circulation. Older plasma lipoproteins are known to be more susceptible to oxidation, suggesting progression of the oxidation during circulation.[32] Relating to this issue, the possible contribution of remnant lipoproteins to CEOOH formation in TRL is of interest, although the present study is limited to VLDL and IDL. Remnant lipoproteins and bioactive components associated with it are believed to be related to atherogenesis, thus can provide significant predictive value of the cardiovascular risk.[33-35] In previous studies, VLDL-remnant is related to oxidation and atherogenicity.[36] In our lipoprotein separation method using ultracentrifugation, remnant lipoproteins cannot be isolated. However, previous studies showed that remnant lipoproteins are largely distributed in IDL fraction.[37] The present study found the wider distribution of CEOOH molecular species in IDL compared to VLDL, which might suggest a possibility that the IDL fraction contained remnant lipoproteins enriched with CEOOH. It is our next interest to quantify CEOOH in remnant lipoproteins isolated by a reported immunoaffinity technique and compare their levels with those of other TRL, LDL, and HDL.[38]

This study revealed several molecular species of CEOOH in the plasma, and native VLDL and IDL, which, in turn, became the target of future quantitative study.

For precise and accurate mass spectrometric quantification, we are synthesizing deuterium labeled CEOOH with the targeted structures as internal standards.

2.5. Conclusion

This study identified six molecular species of CEOOH in human plasma, VLDL and IDL using highly sensitive LC/MS. These markers are potentially useful for identification of individuals who are at high risk for future coronary events. Presence of CEOOH in VLDL and IDL might support the atherogenicity of TRL. Further work is needed to explore the possible underlying pathology of CEOOH in TRL and atherosclerosis. We plan on conducting future experiments to develop methodology for CEOOH quantification and its significance in various clinical conditions.

Tables and Figures

Table 2.1 Diagnostically significant ions of CEOOH in the synthetic standards, VLDL, IDL and plasma obtained from spectra by LC/LTQ Orbitrap in positive-ion mode

			Synthetic		**VLDL**		**IDL**		**Plasma**	
CEOOH Molecular species	Elemental Composition	Theoretical $[M+NH_4]^+$ *(m/z)*	Experimental $[M+NH_4]^+$ *(m/z)*	Mass accuracy (ppm)	Experimental $[M+NH_4]^+$ *(m/z)*	Mass accuracy (ppm)	Experimental $[M+NH_4]^+$ *(m/z)*	Mass accuracy (ppm)	Experimental $[M+NH_4]^+$ *(m/z)*	Mass accuracy (ppm)
Ch18:1-OOH	$C_{45}H_{82}NO_4^+$	700.6238	700.6257	2.71	700.6265	3.85	700.6271	4.71	700.6271	4.71
Ch18:2-OOH	$C_{45}H_{80}NO_4^+$	698.6082	698.6086	0.57	698.6088	0.86	698.6089	1.00	698.6085	0.43
Ch18:3-OOH	$C_{45}N_{78}NO_4^+$	696.5925	696.5922	-0.43	696.5936	1.58	696.5926	0.14	ND	-
Ch20:4-OOH	$C_{47}H_{80}NO_4^+$	722.6082	-	-	722.6088	0.83	722.6085	0.42	722.6089	0.97
Ch20:5-OOH	$C_{45}H_{78}NO_4^+$	720.5925	-	-	720.5902	-3.19	720.5903	-3.05	720.5901	-3.33
Ch22:6-OOH	$C_{49}H_{80}NO_4^+$	746.6082	-	-	746.6087	0.67	746.6061	-2.81	746.6088	0.80

Mass tolerance within ± 5.0 ppm. ND: Not detected.

Table 2.2 Distribution of CEOOH species in VLDL, IDL and plasma

CEOOH Species	VLDL (n=6)	IDL (n=6)	Plasma (n=6)
Ch18:1-OOH	3	2	5**
Ch18:2-OOH	4	6	3*
Ch18:3-OOH	2	4	ND
Ch20:4-OOH	6	6	4*
Ch20:5-OOH	5	6	6**
Ch22:6-OOH	5	6	6**

ND : not detected.

*Detected in fresh plasma samples (n=3).

**Constantly detected in all fresh plasma samples.

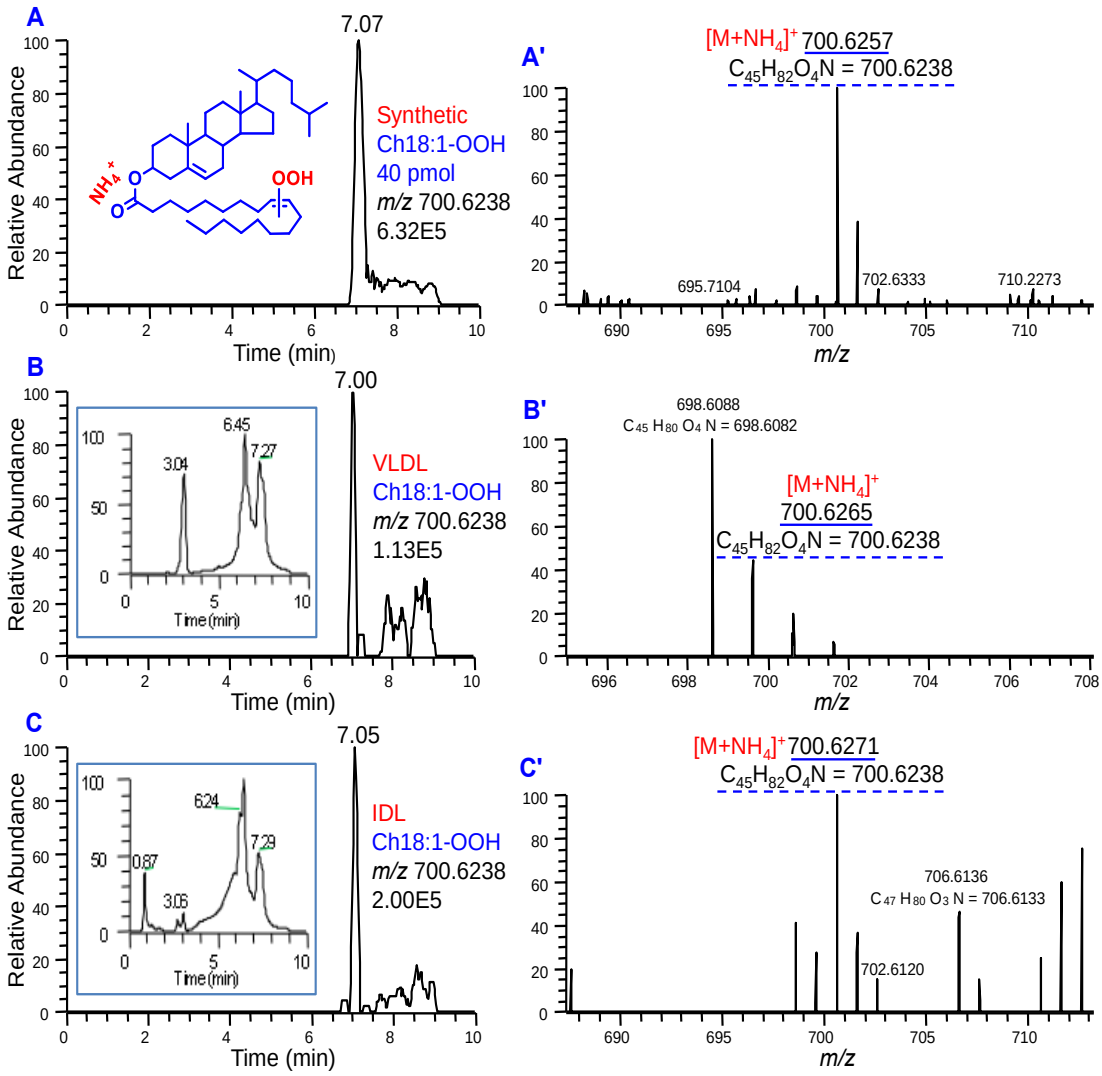

Figure 2.1 LC/LTQ Orbitrap profiles of cholesteryl oleate monohydroperoxide (Ch18:1-OOH) in positive-ion mode. (A) extracted ion (*m/z* 700.6238) chromatogram of synthetic Ch18:1-OOH; (A') mass spectrum of peak associated with retention time at 7.07 min in (A); (B) total ion chromatogram (inside square) and extracted ion (*m/z* 700.6238) chromatogram of VLDL; (B') mass spectrum of peak associated with retention time at 7.00 min in (B)= Ch18:1-OOH; (C) total ion chromatogram (inside square) and extracted ion (*m/z* 700.6238) chromatogram of IDL; (C') mass spectrum of peak associated with retention time at 7.05 min in (C)= Ch18:1-OOH

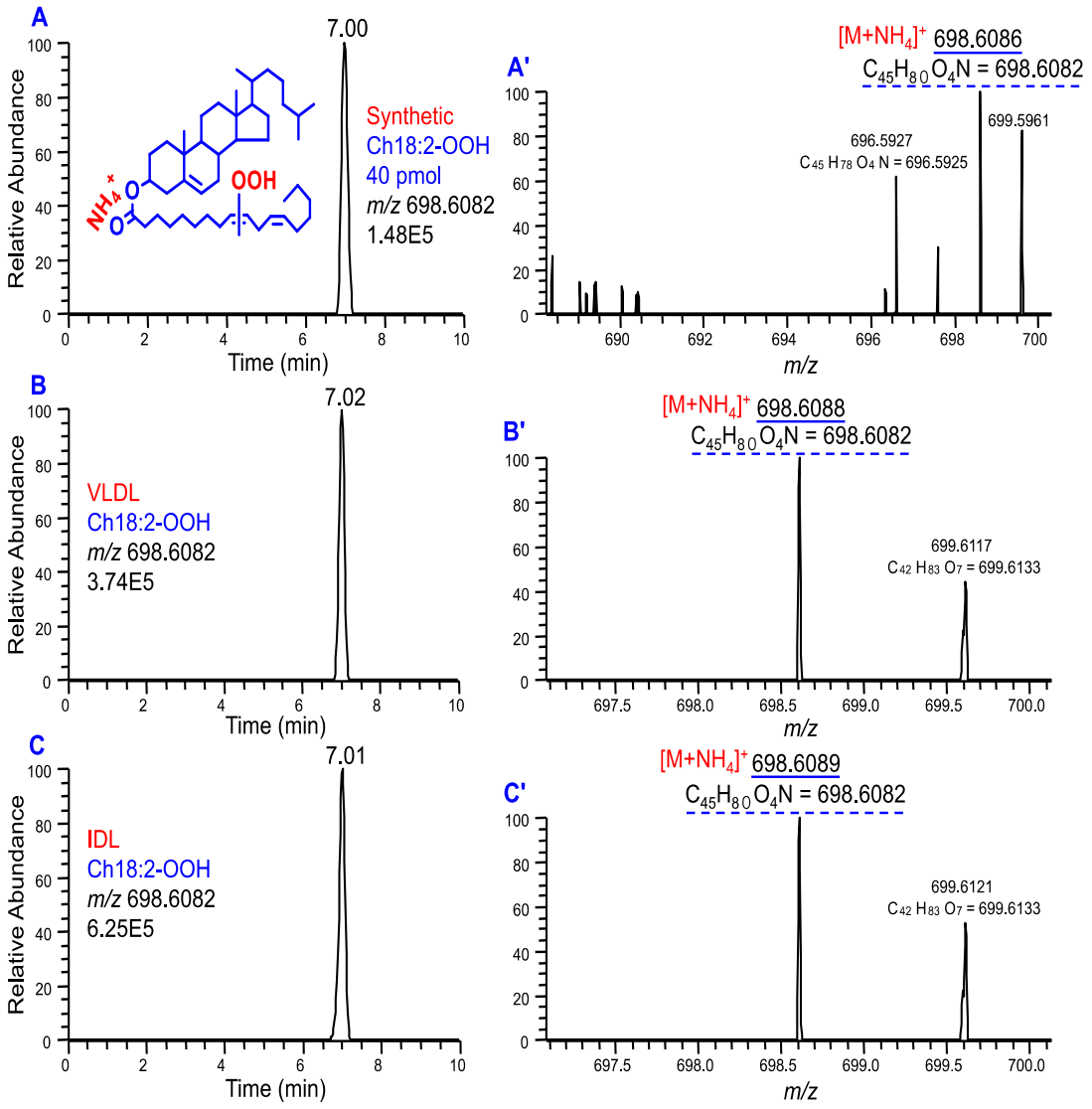

Figure 2.2 LC/LTQ Orbitrap profiles of cholesteryl linoleate monohydroperoxide (Ch18:2-OOH) in positive-ion mode. (A) extracted ion (*m/z* 698.6082) chromatogram of synthetic Ch18:2-OOH; (A') mass spectrum of peak associated with retention time at 7.00 min in (A); (B) extracted ion (*m/z* 698.6082) chromatogram of VLDL; (B') mass spectrum of peak associated with retention time at 7.02 min in (B)= Ch18:2-OOH; (C) extracted ion (*m/z* 698.6082) chromatogram of IDL; (C') mass spectrum of peak associated with retention time at 7.01 min in (C)= Ch18:2-OOH

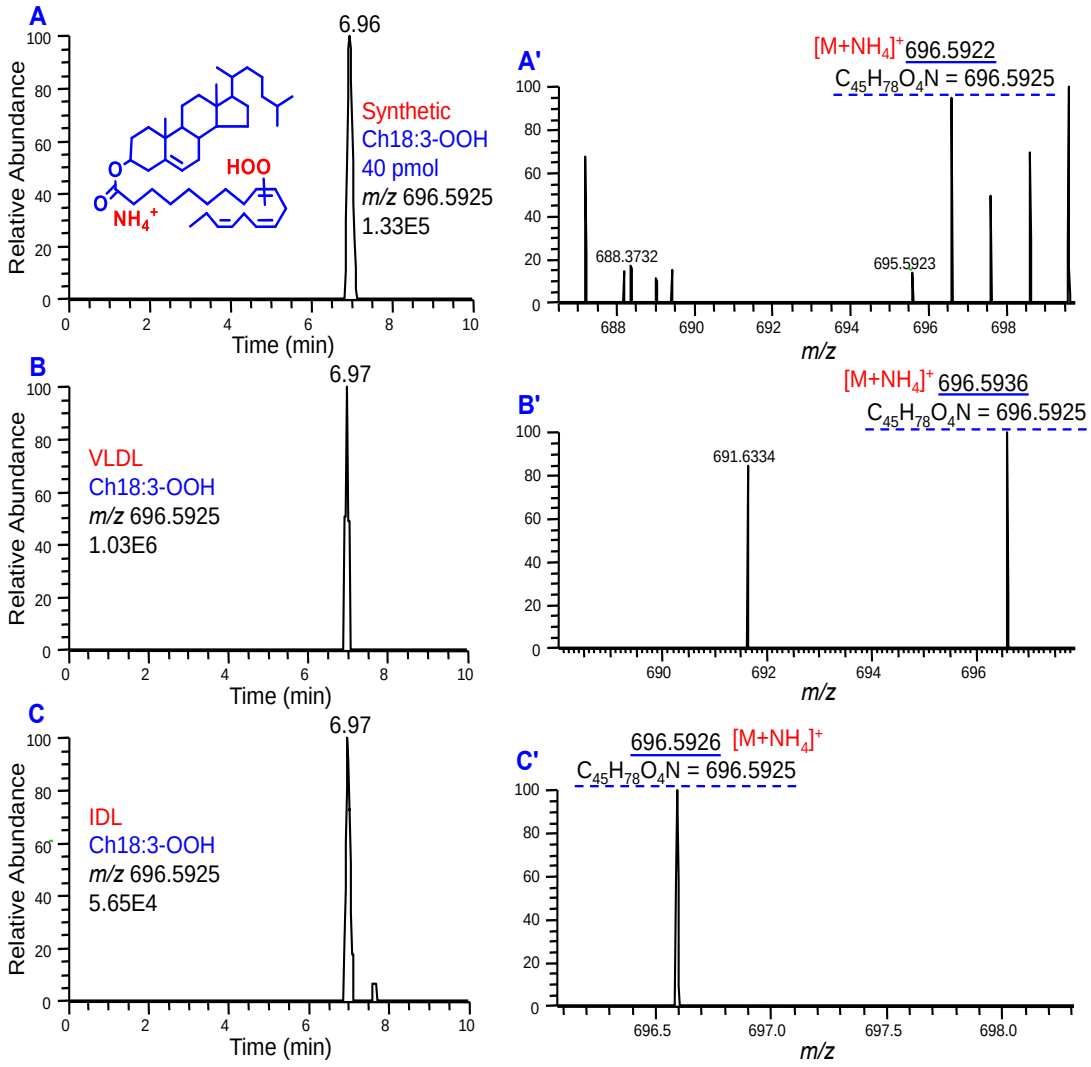

Figure 2.3 LC/LTQ Orbitrap profiles of cholesteryl linolenate monohydroperoxide (Ch18:3-OOH) in positive-ion mode. (A) extracted ion (*m/z* 696.5925) mass chromatogram of synthetic Ch18:3-OOH; (A') mass spectrum of peak associated with retention time at 6.96 min in (A); (B) extracted ion (*m/z* 696.5925) mass chromatogram of VLDL; (B') mass spectrum of peak associated with retention time at 6.97 min in (B)= Ch18:3-OOH; (C) extracted ion (*m/z* 696.5925) mass chromatogram of IDL; (C') mass spectrum of peak associated with retention time at 6.97 min in (C)= Ch18:3-OOH

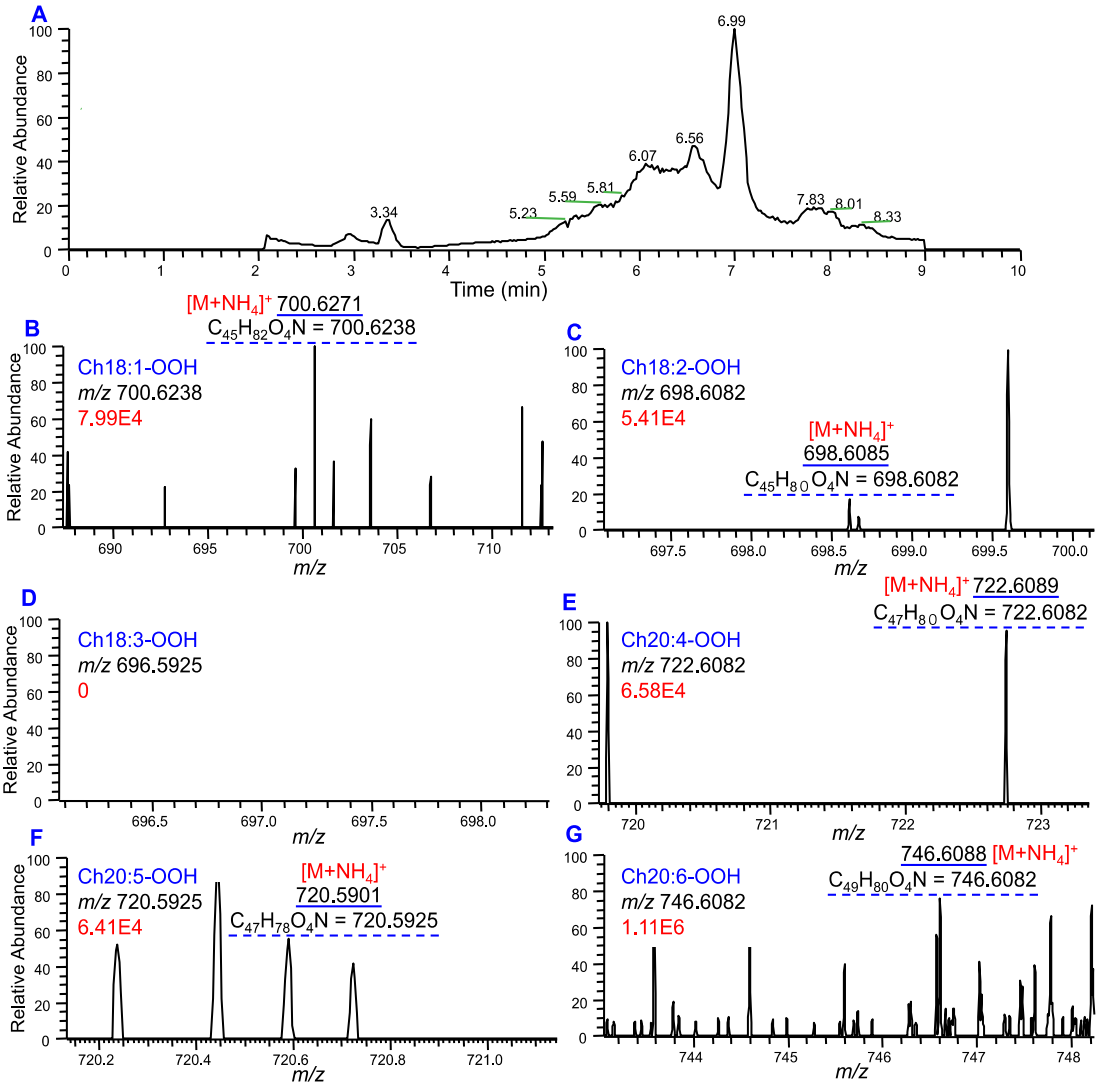

Figure 2.4 LC/LTQ Orbitrap profiles of cholesteryl ester hydroperoxides present in a plasma sample in positive-ion mode. (A) total ion chromatogram of plasma extract; (B) mass spectrum showing *m/z* 700.6271 = Ch18:1-OOH; (C) mass spectrum showing *m/z* 698.6085 = Ch18:2-OOH; (D) Ch18:3-OOH (*m/z* 696.5925) is absent in plasma; (E) mass spectrum showing *m/z* 722.6089 = Ch20:4-OOH; (F) mass spectrum showing *m/z* 720.5901 = Ch20:5-OOH; (G) mass spectrum showing *m/z* 746.6088 = Ch20:6-OOH

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Chapter 3: Identification of Molecular Species of Oxidized Triglyceride in Plasma and its Distribution in Lipoproteins

3.1. Introduction

A large number of epidemiological, clinical and experimental studies continue to support both fasting and non-fasting (postprandial) plasma triglycerides as an independent risk factor for cardiovascular diseases.[1-7] The majority of the plasma triglycerides are carried in TRL with density <1.019 kg/L that includes chylomicrons, VLDL, and IDL. A growing body of evidence supports association of the TRL with atherosclerosis and CHD. Evidence of atherogenicity of these TRL, particularly VLDL and IDL have been well documented.[8-11] However, whether triglycerides itself is causative or the metabolic changes that it brings, for example, reduction of HDL or its indirect reflection of remnant lipoproteins remain to be fully elucidated. Therefore, the specific role of triglycerides in TRL towards the progression of CHD is uncertain and has long been controversial.[12-14]

Oxidative modification, including peroxidation of lipid content in lipoproteins is believed to play a crucial role in the atherosclerotic process. Oxidized lipids in the TRL may be one of the promoting factors for its atherogenicity.[15] As hydroperoxides are the major reaction products of lipid peroxidation,[16] its identification in the TRL can uncover the possible association of TRL in the atherogenesis. Most analytical

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approaches of lipid peroxidation have used nonspecific methods for total FA hydroperoxide (FAOOH) products. The direct measurements of the hydroperoxides were mainly focused towards CE[17] and phospholipids.[18] In contrast, the identification of oxidized triglycerides and their possible associations with CHD were largely ignored.

The HPLC approach was unable to detect TGOOH in plasma, adipose tissue, and lipoprotein fraction including chylomicron and VLDL; even after the test animals were fed with oxidized oils.[19,20] Several investigators have identified TGOOH species in artificially-oxidized oils.[21,22] The identification of molecular species of oxidized lipids has been largely limited in artificially-oxidized lipoproteins. Hui et al. have recently identified several molecular species of CEOOH and TGOOH in artificially-oxidized lipoprotein fractions using highly sensitive reversed-phase LC/LTQ Orbitrap.[17,23]

This study described a method for detection of TGOOH in biological samples and identified its several molecular species in plasma. The lipoprotein origin of the detected TGOOH was also investigated.

3.2. Materials and Methods

3.2.1. Chemicals

1-Oleoyl-2-linoleoyl-3-palmitoylglycerol monohydroperoxide (TGOOH-18:1/18:2/16:0), 1,2-dioleoyl-3-palmitoylglycerol monohydroperoxide (TGOOH-18:1/18:1/16:0), and triolein monohydroperoxide (TGOOH-18:1/18:1/18:1) were synthesized chemically by method reported elsewhere.[24,25] Unless specified, all other chemicals and solvents were of analytical grade obtained from Wako Pure Chemical Industry (Osaka, Japan).

3.2.2. Specimens

This study was conducted in nine apparently healthy human volunteers (6 men and 3 women; mean age±SD, 29.1±5.7 years, 23–40 years) after written informed consent. To minimize the influence of dietary oxidized triglycerides, EDTA blood samples (15 mL) were collected after 14–16 hours of fasting. All of the remaining procedure on the samples was done at temperature below 4°C and every effort was made to prevent auto-oxidation of lipids during the specimen processing. The plasma samples were stored at -80°C for no longer than 3 months. Furthermore, we collected additional blood samples from three of the nine donors and analyzed freshly by the LC/LTQ Orbitrap to rule out possible auto-oxidation during storage.

3.2.3. Isolation of Lipoproteins

The plasma samples were processed within 1 hour after collection for the isolation of lipoproteins. VLDL and IDL were isolated from the plasma by sequential ultracentrifugation in KBr (Optima MAX, Beckman Coulter, Fullerton, CA, USA). The detail procedure of the isolation has been described elsewhere.[26] Briefly, plasma (2.0 mL) was mixed with 6.0 mL of d=1.006 kg/L solution containing EDTA-2Na (0.01%, w/v) to prevent oxidative process during ultracentrifugation. Upper fraction (2.5 mL) was collected as VLDL after ultracentrifugation at 50,000 rpm for 14 hours at 4°C in a near-vertical tube rotor (MLN-80, Beckman Coulter, Fullerton, CA, USA). The remaining fraction was adjusted to d=1.019 kg/L with KBr solution (2.5 mL) and centrifuged at 40,000 rpm for 20 hours at 4°C, followed by the collection of upper fraction (2.5 mL) as IDL. The remaining fraction was then adjusted to d=1.225 kg/L and centrifuged at 50,000 rpm for 20 hours at 4°C. The upper fractions (d= 1.019-1.225 kg/L) were concentrated and used for further isolation of LDL and HDL by

high-performance size exclusion chromatography with Superose 6 column (GE Healthcare, Uppsala, Sweden). The column was eluted with isocratic flow of 50 mmol/L phosphate buffer (pH 7.4) containing 150 mmol/L NaCl and 1 mmol/L EDTA at a rate of 0.5 mL/min and 4°C in Shimadzu Prominence LC-20AD HPLC (Kyoto, Japan). The isolated individual lipoproteins were concentrated using XM-50 and Millipore Amicon Bioseparation Stirred Cells (Thermo Fisher Scientific, Waltham, MA, USA) and further purified using centrifugal filter devices [Ultracel - 50K for HDL and Ultracel 100K for other lipoproteins] (Merck Millpore Ltd., Carrigtwohill, Ireland). Throughout the process of the isolation and purification of lipoproteins, EDTA containing buffer was used and the temperature was maintained at 4°C to ensure prevention of its oxidation. The isolated lipoproteins were stored at -80°C for no longer than 3 months, until mass spectrometric analysis.

3.2.4. Determination of Lipoproteins Composition

The molecular composition of the lipoproteins was determined by measuring total and free cholesterol, triglycerides and phospholipids by enzymatic methods (Kyowa Medex Co, Ltd., Tokyo, Japan) in Hitachi 7170 (Tokyo, Japan). Protein content in the lipoproteins was measured by modified Lowry method.[27]

3.2.5. Purity Assessment of the Lipoproteins

The characteristic motility of lipoproteins was confirmed in polyacrylamide gel disc electrophoresis (LipoPhor, Jokoh Co, Tokyo, Japan). Briefly, 200 μL of Sudan black B dye containing loading gel and 25 μL of serum or lipoproteins with each total lipid concentration of 4.0 μg/μL were applied to precast gel tube. After photo-polymerization of loading gel by exposing to the light from a fluorescent lamp for 40

min, electrophoresis was carried out in a pH 7.4 buffer (Tris 50 mmol/L, boric acid 50 mmol/L) at 5 mA/per tube and 150 V for approximately 30 min. Apolipoproteins distribution of each lipoprotein were demonstrated in 3-10% SDS-PAGE (ATTO Bioscience and biotechnology, Tokyo, Japan). Ten μL of lipoproteins (0.5 μg/μL protein) was mixed with 10 μL of sample buffer containing 2-mercaptoethanol and heated at 95°C for 5 min. Ten μL of the mixture was applied to each assigned well and stained with SimpleBlue™ SafeStain-Coomassie Brilliant Blue (Invitrogen, USA). Lipoproteins from three donors were excluded from this study due to either inadequate recovery or impurity in isolated lipoproteins.

3.2.6. Preparation of Sample for LC/MS

Total lipids were extracted from the plasma (n=12; 9 stored and 3 fresh) and the lipoproteins (n=6; 4 men and 2 women) by the method previously described.[26] Briefly, 0.1 mL of lipoproteins (0.5 μg/μL of total lipids) or 0.2 mL of plasma was mixed with 0.4 mL of freshly prepared 0.005% (w/v) 2,6-di-*tert*-butyl-*p*-cresol [Butylated hydroxytoluene (BHT), as an antioxidant] in acetonitrile and 2.0 mL of chloroform. We decreased the volume of lipoprotein solutions compared to our original reported method.[17,18] However, because of the hydrophobic nature of triglycerides, this modification in the volume of aqueous phase had no effect on the performance of lipid extraction. The content was thoroughly mixed and centrifuge at 3000 *g* for 10 min at 4°C, followed by collection of the chloroform extract. The chloroform extract was repeated twice to ensure complete extraction of lipids. The chloroform extract was then completely evaporated *in vacuo* (Model CC-105, TOMY Digital Biology, Tokyo, Japan) and the residue was reconstituted with 300 μL of methanol. Ten μL of the extract was injected for LC/LTQ Orbitrap analysis.

3.2.7. LC/LTQ Orbitrap

Reserved-phase liquid chromatographic separation was performed on a Syncronis C18 column [50 mm × 2.1 (i.d.) mm; particle size 1.7 μm] (Thermo Fisher Scientific, Waltham, MA, USA) at 60°C. The HPLC gradient elution program was same as described in chapter 1. High-resolution mass spectrometric analysis was performed using LTQ XL Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). ESI tandem mass spectrometry analysis was performed in positive-ion mode and mass spectra were obtained in Fourier-transform mode. TGOOH was detected as the $[M+NH_4]^+$. EIC were drawn with the mass tolerance set at 5.0 ppm. Data processing was done in Qual Browser 2.0 software (Thermo Fisher Scientific, Waltham, MA, USA). Each spectrum was converted into a peak list reporting *m/z*, relative intensity and the sum composition for each peak detected with a signal-to-noise ratio above 6.

3.2.8. Quantification of TGOOH

The relative concentration of TGOOH was determined by single point calibration using synthetic TGOOH as external standards. The standards of TGOOH were dissolved in methanol (2 pmol/μL) and 20 μL was injected into the LC/LTQ Orbitrap mass spectrometer. The concentration of TGOOH-18:1/18:2/16:0, TGOOH-18:1/18:1/16:0, and TGOOH-18:1/18:1:18:1 in the samples were calculated by comparing the peak area of detected TGOOH to the peak area generated by known concentration of the corresponding TGOOH standards. TGOOH-18:1/18:1/18:1 was used as reference standard for the measurement of remaining TGOOH, that is, TGOOH-16:0/18:2/16:0, TGOOH-16:0/18:1/16:0, TGOOH-18:1/18:2/18:1, TGOOH-16:0/20:5/16:0, TGOOH-16:0/20:4/16:0, TGOOH-16:0/20:5/18:1, TGOOH-

16:0/20:4/18:1, and TGOOH-16:0/22:6/18:1. The total triglycerides in the samples were measured by an enzymatic method (Kyowa Medex Co, Ltd., Tokyo, Japan) in Hitachi 7170 (Tokyo, Japan) automated analyzer. The relative TGOOH concentration was expressed as the ratio of each TGOOH quantity (μmol) to the total triglycerides (mol) present in the sample. The sum of concentrations of each molecular species of TGOOH detected in a sample comprises total TGOOH.

3.3. Results

Serum cholesterol (Total, Free, LDL and HDL), triglycerides, and phospholipids were within the normal range in all of the study participants (Table 3.1). We isolated each lipoprotein fractions with high degree of purity as determined by its molecular composition (Table 3.2), characteristic mobility in LipoPhor and apolipoprotein composition determined by SDS-PAGE (Figure 3.1). Mean percentage content of triglycerides in VLDL and IDL were 61.8% and 31.4%, respectively. VLDL and IDL contained Apo B-100 (MW 512 kDa), Apo E (MW 34 kDa) and Apo C (MW 9 kDa). Based on size and density, each lipoprotein shows characteristic motility on the polyacrylamide gel disc electrophoresis (Figure 3.1B).

3.3.1. Detection of TGOOH in Plasma

LC/LTQ Orbitrap in positive-ion mode was used for analysis of TGOOH in the extract of lipoproteins and plasma. We detected and identified 10 molecular species of TGOOH in the plasma. TGOOH-18:1/18:1/16:0 and TGOOH-16:0/18:2/16:0 were present in all plasma. TGOOH-18:1/18:2/16:0, TGOOH-16:0/20:5/16:0, TGOOH-16:0/20:4/16:0 and TGOOH-16:0/20:4/18:1 were found in most of the plasma (Table 3.3). In contrast, TGOOH-16:0/22:6/18:1 was not detected in any plasma sample.

Mean concentration of plasma TGOOH was 56.1±25.6 µmol per mol of triglycerides. TIC and spectra of a plasma extract are shown in Figure 3.2.

The freshly analyzed plasma (n=3) shows exactly similar distribution of TGOOH as that of plasma stored at -80°C. Moreover, the relative concentrations of TGOOH were comparable between the fresh and frozen plasma (mean ±SD; 45.9 ±29.6 vs 48.1 ±30.9 µmol/mol triglycerides, respectively).

3.3.2. Detection of TGOOH in Lipoproteins

Ten molecular species of TGOOH were found in VLDL and IDL but as expected, no TGOOH were detected in either LDL or HDL.

Figure 3.3A-C shows extracted ion chromatograms (EIC) of *m/z* 906.7756 in positive-ion mode for synthetic TGOOH-18:1/18:2/16:0 (40 pmol), VLDL and IDL respectively; their corresponding mass spectra are shown in Figure 3.3A'-C'. The synthetic TGOOH-18:1/18:2/16:0 eluted at RT of 6.92 min and its corresponding mass spectrum shows $[M+NH_4]^+$ at *m/z* 906.7762 (elemental composition $C_{55}H_{104}O_8N$, theoretical mass 906.7756). The TIC of VLDL and IDL extract show absence of clearly defined HPLC peaks (Figure 3.3B and 3.3C). However, extraction of a particular signal, *m/z* 906.7756, from the TIC led to a defined peak. A single peak was observed at RT of 6.95 min, and the corresponding mass spectrum obtained is shown in Figure 3.3B'. The base peak observed at *m/z* 906.7743 corresponding to $[M+NH_4]^+$, and the ion has the same elemental composition and theoretical mass as the ion from synthetic TGOOH-18:1/18:2/16:0, thus the peak at RT of 6.95 min in Figure 3.3B was identified as TGOOH-18:1/18:2/16:0 in VLDL. In the same way, the peak at RT of 6.93 min in Figure 3.3C obtained from IDL was identified as TGOOH-18:1/18:2/16:0.

Chromatogram and mass spectrum of TGOOH-18:1/18:1/16:0 obtained from standard, VLDL and IDL are shown in Figure 3.4. Standard TGOOH-18:1/18:1/16:0 was eluted at the RT of 6.95 min (Figure 3.4A), and the corresponding mass spectrum revealed $[M+NH_4]^+$ at *m/z* 908.7916 (elemental composition $C_{55}H_{106}O_8N$, theoretical mass 908.7913) (Figure 3.4A'). In the Figure 3.4B' and 3.4C', the base peak at *m/z* 908.7950 and *m/z* 908.7958 corresponded to $[M+NH_4]^+$ have the same elemental composition and theoretical mass as the ions from standard TGOOH-18:1/18:1/16:0 (Figure 3.4A'), implying the peak at RT of 6.95 min in Figure 3.4B and Figure 3.4C were of TGOOH-18:1/18:1/16:0 in VLDL and IDL, respectively.

Figure 3.5A-C shows EIC of *m/z* 934.8069 for synthetic TGOOH-18:1/18:1/18:1 (40 pmol), VLDL, and IDL; and their corresponding mass spectra are shown in Figure 3.5A'-3.5C', respectively. The synthetic TGOOH-18:1/18:1/18:1 was eluted at the RT of 7.02 min (Figure 3.5A); the corresponding mass spectrum is shown in Figure 3.5A', elucidating $[M+NH_4]^+$ at *m/z* 934.8069 (elemental composition $C_{57}H_{108}O_8N$, theoretical mass 934.8069). In the Figure 3.5B', a peak at *m/z* 934.8071 corresponding to $[M+NH_4]^+$ have the same elemental composition and theoretical mass as the ions from synthetic TGOOH-18:1/18:1/18:1, indicating that the peak at RT of 7.06 min in Figure 3.5B obtained from VLDL extract was of TGOOH-18:1/18:1/18:1. Similarly, TGOOH 18:1/18:1/18:1 was also identified in IDL.

TGOOH-16:0/18:2/16:0, TGOOH-18:1/18:2/18:1, TGOOH-16:0/20:5/16:0, TGOOH-16:0/20:4/16:0, TGOOH-16:0/20:5/18:1, TGOOH-16:0/20:4/18:1, and TGOOH-16:1/22:6/18:1 were also identified in VLDL and IDL. All characteristic ions of TGOOH detected in VLDL, IDL and plasma in a sample are summarized in Table 3.4.

3.3.3. Distribution of TGOOH

The present study identified 11 TGOOH molecular species in overall. The distribution of these TGOOH and their relative concentration were shown in Table 3.3. TGOOH-16:0/18:2/16:0 is the most predominant TGOOH consistently detected in all of VLDL, IDL and plasma. Beside this TGOOH-18:1/18:2/16:0, TGOOH-18:1/18:1/18:1, TGOOH-18:1/18:2/18:1, TGOOH-16:0/20:4/16:0, TGOOH-16:0/20:5/18:1, TGOOH-16:0/20:4/18:1 and TGOOH-16:0/22:6/18:1 were detected in all VLDL and IDL. We did not observe any differences in TGOOH distribution between VLDL and IDL. However, although mean percentage content of triglycerides in IDL is almost half of VLDL, the mean concentration of TGOOH is higher in IDL compared to that of VLDL (Table 3.2).

3.4. Discussion

This is the first study to elucidate the presence of oxidized triglycerides in human plasma and lipoproteins in its native form. Most of the previous studies have used artificially-oxidized plasma or lipoproteins to demonstrate oxidized lipids. However, such artificially-oxidized conditions do not necessarily mimic the real physiological condition. Use of highly sensitive reversed-phase liquid chromatography with a hybrid linear ion trap Orbitrap mass spectrometer, coupled with authentic standards enabled us to identify 10 TGOOH molecular species in the plasma (Table 3.3), on the basis of their mass spectra and RT on LC.

Triglycerides are absolutely hydrophobic; therefore, the only possible way of its transportation in plasma is through lipoproteins. We also isolated individual lipoproteins, including IDL from the plasma and analyzed it for the detection of TGOOH. We found that TGOOH present in plasma were carried by TRL but not by

LDL and HDL. To minimize the possible influence of dietary oxidized lipids, chylomicrons were not analyzed. Therefore, the major TRL in fasted subjects is VLDL and to a lesser extent IDL. The relative concentration of triglycerides is considerably lower in LDL and HDL than that for TRL (Table 3.2). We suppose that this is the main reason for undetectably low concentration of TGOOH in the LDL and HDL. In this study, LDL and HDL were isolated by HPLC in Superose 6 column to provide better resolution in the separation and also it minimizes the time required for the subsequent sequential ultracentrifugation to separate LDL and HDL. Due to similarity in diameter, however, the size-exclusion HPLC is unable to discriminate IDL from LDL (Figure 3.6). Therefore, VLDL and IDL were isolated by ultracentrifugation. Nonetheless, each isolated lipoprotein fractions were of high purity as confirmed by its chemical composition (Table 3.2), characteristic motility in PAGE and apolipoprotein composition (Figure 3.1). Furthermore, we believe that the process of isolation of lipoproteins and its storage at -80°C do not induce oxidation of triglycerides as both the freshly analyzed and frozen stored plasma samples contain identical TGOOH distribution with comparable concentration of TGOOH.

Previous analytical methods of measuring LOOH were limited to its determination after initiating oxidation *in vitro* or measurement of total cholesteryl ester or phospholipid hydroperoxides.[28-30] Browne et al. reported up to 13 distinct regioisomers of FAOOH on oxidatively modified human plasma using HPLC.[31] However, total lipids were extracted from the plasma therefore, source of these FAOOH, whether it is derived from phospholipids, CE, triglycerides or free FA was not investigated individually. In addition, previous studies have remarked that the plasma hydroperoxides are mainly confined to CE or phospholipids.[24,32] Several investigators were unable to detect TGOOH in plasma and lipoprotein fractions.[33,34]

Furthermore, Suomela et al. reported that TGOOH was not detected in pig plasma, small intestine epithelial cell and adipose tissue even they were fed with a diet rich in oxidized triacylglycerol.[19,20,35] Our successful demonstration of TGOOH in VLDL and IDL is due to the high sensitivity of LC/LTQ Orbitrap, which can obtain accurate *m/z* values for adducts of molecular ions from individual molecules by high mass resolution. It provides high resolution EIC within ±5.0 ppm relative mass deviation and spectra with selected extraction of ions at ±5.0 ppm accuracy. The lower limit of detection of this analytical approach for each synthesized TGOOH was 0.1 pmol per injection (S/N=10:1).

The physiologically relevant mechanisms of existence of TGOOH in TRL are not clearly understood. Although the VLDL particles carry mainly endogenous triglycerides synthesized *de novo* in the liver, esterification of oxidized FA from diets into triglycerides during its assembly in the liver cannot be excluded.[36,37] Moreover, the oxidation of triglycerides may occur *in situ* in the circulation due to interaction with ROS. The VLDL-triglycerides undergo hydrolysis by lipoprotein lipase and are serially converted to VLDL remnants to IDL, yet relatively rich in triglycerides.[38] We did not observe any difference in distribution of TGOOH between VLDL and IDL. However, interestingly, we found that the relative concentration of TGOOH is higher in IDL compared to VLDL. Older plasma lipoproteins are more susceptible to oxidation; therefore, triglycerides in the VLDL remnants and IDL, though present in relatively low amount, are at more risk of getting oxidized.[39] Liver-derived VLDL remnants rather than chylomicrons are the major contributors to remnant lipoprotein-triglycerides and reflect postprandial hypertriglyceridemia.[40,41] In our method of lipoproteins separation by ultracentrifugation, VLDL remnants are indistinguishable from IDL and are largely distributed in IDL fraction.[42] Therefore, it is likely that the

TGOOH detected in the IDL fraction are those from VLDL remnants. It is our next interest to quantify TGOOH in remnant lipoproteins isolated by immunoaffinity technique. Regardless, TGOOH present in the VLDL and its remnants, and possibly IDL may participate actively in the development of atherosclerosis.

Although the role of TRL in atherosclerosis is poorly understood, an increasing body of evidence supports its atherogenicity. Both VLDL and IDL can contribute to the formation of foam cells, a hallmark of early atherosclerosis and oxidized forms of these lipoproteins have been identified in atherosclerotic lesions.[8,43] *In vitro* studies have shown that lipolysis of TRL leads to release a number of potentially toxic oxidized FA and elicit inflammatory responses.[44,45] When the TGOOH containing TRL are hydrolyzed by lipoprotein lipase, an enzyme anchored to endothelial cells, the artery walls are constantly exposed to the liberated oxidized FA. Therefore, it appears that significant amounts of oxidized FA are released during lipolysis of TRL and perhaps this phenomenon may result endothelial dysfunction and inflammation, another key feature in early atherosclerosis. Furthermore, previously, we have demonstrated both the VLDL and IDL carry CEOOH (See chapter 2), which further adds to atherogenicity of TRL. Our study was largely limited to identify oxidized triglycerides in the lipoproteins; therefore, exploration of its association with the atherosclerotic process is beyond the objective of this study. Further works are needed to find the association of TGOOH in atherosclerotic process and determine the clinical utility of TGOOH measurement in assessment of cardiovascular risk, and are of future interest. However, demonstration of the existence of oxidized triglycerides in the VLDL and IDL may provide new insight to understand pathogenicity of these TRL. Another limitation of this study is that we determined the relative concentration of TGOOH on the basis of the peak intensity of external standards. The accurate

measurement of absolute TGOOH concentration may require selective reaction monitoring (SRM) in the mass spectrometer and appropriate internal standards. It is our future plan to develop quantitative TGOOH assay with SRM in LC-MS/MS and determine its clinical utility in various disease conditions.

3.5. Conclusion

This study identified 11 molecular species of TGOOH in human plasma, VLDL and IDL using LC/MS. Presence of TGOOH in the VLDL and IDL may contribute to the atherogenicity of TRL. Further work is needed to elucidate the association of the oxidized triglyceride in atherosclerosis.

Tables and Figures

Table 3.1 Basic characteristics of study participants

	Mean±SD (n=9)
Age (Years)	29.1±5.7
BMI (Kg/m2)	20.8±2.8
Free cholesterol (mmol/L)	1.4±0.2
Total cholesterol (mmol/L)	4.5±0.7
LDL-cholesterol (mmol/L)	2.7±0.8
HDL-cholesterol (mmol/L)	1.5±0.4
Triglycerides (mmol/L)	0.8 ±0.4
Phospholipids (mmol/L)	2.9±0.3

Table 3.2 Chemical composition of human plasma lipoproteins

	VLDL Mean±SD (n=6)	IDL Mean±SD (n=6)	LDL Mean±SD (n=6)	HDL Mean±SD (n=6)
Surface components				
Cholesterol (%)	5.3±0.4	8.1±0.8	10.3±0.4	3.9±0.5
Phospholipids (%)	16.3±1.6	21.8±1.2	23.3±2.3	30.0±4.6
Apolipoproteins (%)	8.0±2.2	16.0±2.8	23.2±2.5	41.6±3.8
Core lipids				
Triglycerides (%)	61.8±3.3	31.4±3.1	4.3±0.5	2.6±1.0
Cholesteryl esters (%)	8.7±0.7	22.7±1.9	38.9±5.2	21.9±4.1
TGOOH (μmol/mol TG)	349.8±253.6	512.5±173.2	ND	ND

VLDL: Very low-density lipoprotein; IDL: Intermediate-density lipoprotein; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; TG: Triglycerides; TGOOH: Triglyceride hydroperoxides; ND: Not detected

Table 3.3 Distribution and relative concentration of TGOOH species in VLDL, IDL and Plasma.

TGOOH species	VLDL (N=6) Mean ±SD* (n)	IDL (N=6) Mean ±SD* (n)	Plasma (N=9) Mean ±SD* (n)
18:1/18:2/16:0	64.4±47.4 (6)	80.0±38.1 (6)	13.1±9.8 (6)
18:1/18:1/16:0	6.0±4.0 (3)	12.2±17.2 (3)	7.8±5.2 (9)
18:1/18:1/18:1	9.1±10.0 (6)	27.2±20.0 (6)	16.1±0.9 (2)
16:0/18:2/16:0	13.6±7.6 (6)	35.3±32.5 (6)	10.9±6.3 (9)
16:0/18:1/16:0	ND	ND	2.7±2.0 (4)
18:1/18:2/18:1	38.4±35.4 (6)	81.1±66.9 (6)	28.4±9.9 (2)
16:0/20:5/16:0	4.9±4.8 (4)	7.6±8.4 (4)	4.5±4.2 (5)
16:0/20:4/16:0	75.7±52.8 (6)	98.0±30.0 (6)	10.9±5.4 (8)
16:0/20:5/18:1	44.6±35.3 (6)	53.9±24.9 (6)	3.7±2.1 (3)
16:0/20:4/18:1	46.6±37.0 (6)	56.7±27.9 (6)	7.3±3.8 (5)
16:0/22:6/18:1	51.1±51.2 (6)	69.2±60.7 (6)	ND

N: total number of samples; (n): number of samples in which TGOOH molecule is detected; ND: Not detected.
**Concentration expressed in μmol/mol triglycerides.*

Table 3.4 Diagnostically significant ions of TGOOH in the synthetic standards, VLDL, IDL and plasma obtained from spectra by LC/LTQ Orbitrap in positive ion mode

TGOOH Molecular species	Element composition	Theoretical	Synthetic		VLDL		IDL		Plasma	
			Experimental	Mass accuracy	Experimental	Mass accuracy	Experimental	Mass accuracy	Experimental	Mass accuracy
		$[M+NH_4]^+$ *(m/z)*	$[M+NH_4]^+$ *(m/z)*	(ppm)	$[M+NH_4]^+$ *(m/z)*	(ppm)	$[M+NH_4]^+$ *(m/z)*	(ppm)	$[M+NH_4]^+$ *(m/z)*	(ppm)
18:1/18:2/16:0	$C_{55}H_{104}O_8N^+$	906.7756	906.7762	0.66	906.7743	-1.43	906.7751	-0.55	906.7745	-1.21
18:1/18:1/16:0	$C_{55}H_{106}O_8N^+$	908.7913	908.7916	0.33	908.7950	4.07	908.7958	4.95	908.7925	1.32
18:1/18:1/18:1	$C_{57}H_{108}O_8N^+$	934.8069	934.8069	0	934.8071	0.21	934.808	1.18	ND	-
16:0/18:2/16:0	$C_{53}H_{102}O_8N^+$	880.7600	-	-	880.7621	2.38	880.7626	2.95	880.7621	2.38
16:0/18:1/16:0	$C_{53}H_{104}O_8N^+$	882.7756	-	-	ND	-	ND	-	ND	-
18:1/18:2/18:1	$C_{57}H_{106}O_8N^+$	932.7913	-	-	932.7905	-0.86	932.7908	-0.54	932.7901	-1.29
16:0/20:5/16:0	$C_{55}H_{100}O_8N^+$	902.7443	-	-	902.7451	0.89	902.7451	0.89	902.7449	0.66
16:0/20:4/16:0	$C_{55}H_{102}O_8N^+$	904.7600	-	-	904.7603	0.33	904.7597	-0.33	904.7599	-0.11
16:0/20:5/18:1	$C_{57}H_{102}O_8N^+$	928.7600	-	-	928.7596	-0.43	928.7606	0.65	928.7599	-0.11
16:0/20:4/18:1	$C_{57}H_{104}O_8N^+$	930.7756	-	-	930.775	-0.64	930.7749	-0.75	930.7773	1.83
16:0/22:6/18:1	$C_{59}H_{104}O_8N^+$	954.7756	-	-	954.7755	-0.1	954.7752	-0.42	ND	-

ND: Not detected.

Mass tolerance within ± 5.0 ppm.

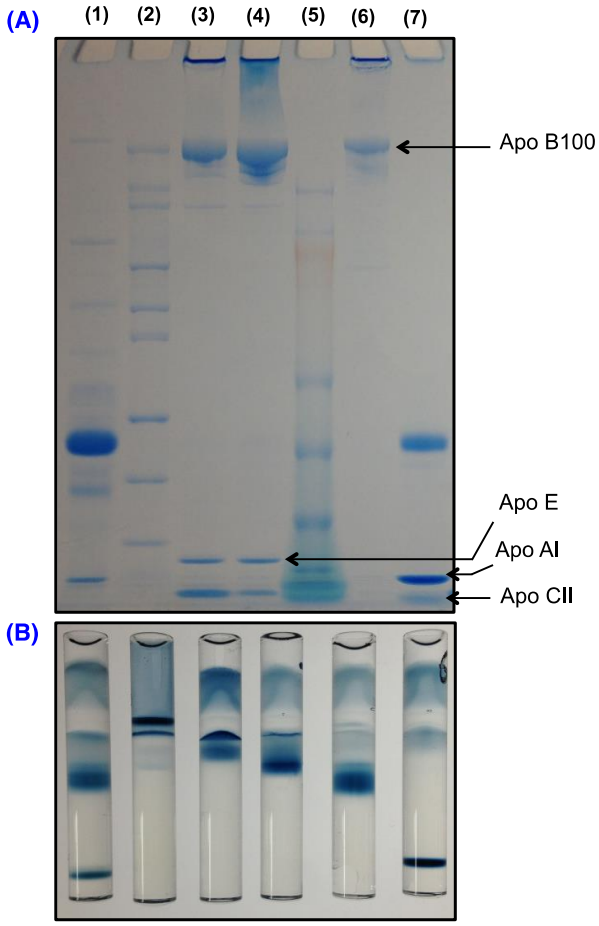

Figure 3.1 (A) SDS-PAGE of apolipoproteins. 1: plasma (1:1000 dilution), 2: HMW*, 3: VLDL, 4: IDL, 5: LMW**, 6: LDL, 7: HDL stained with Coomassie brilliant blue; (B) Polyacrylamide gel disc electrophoresis of plasma and lipoproteins, From left to right as plasma, lipoprotein free plasma, VLDL, IDL, LDL, and HDL. Lipids in each fraction were stained by Sudan black B dye.

**HMW; high molecular weight standard (HiMark unstained HMW protein standard, Invitrogen, USA*

***LMW; low molecular weight standard (SeeBlue plus Pre-stained standard, Invitrogen, USA)*

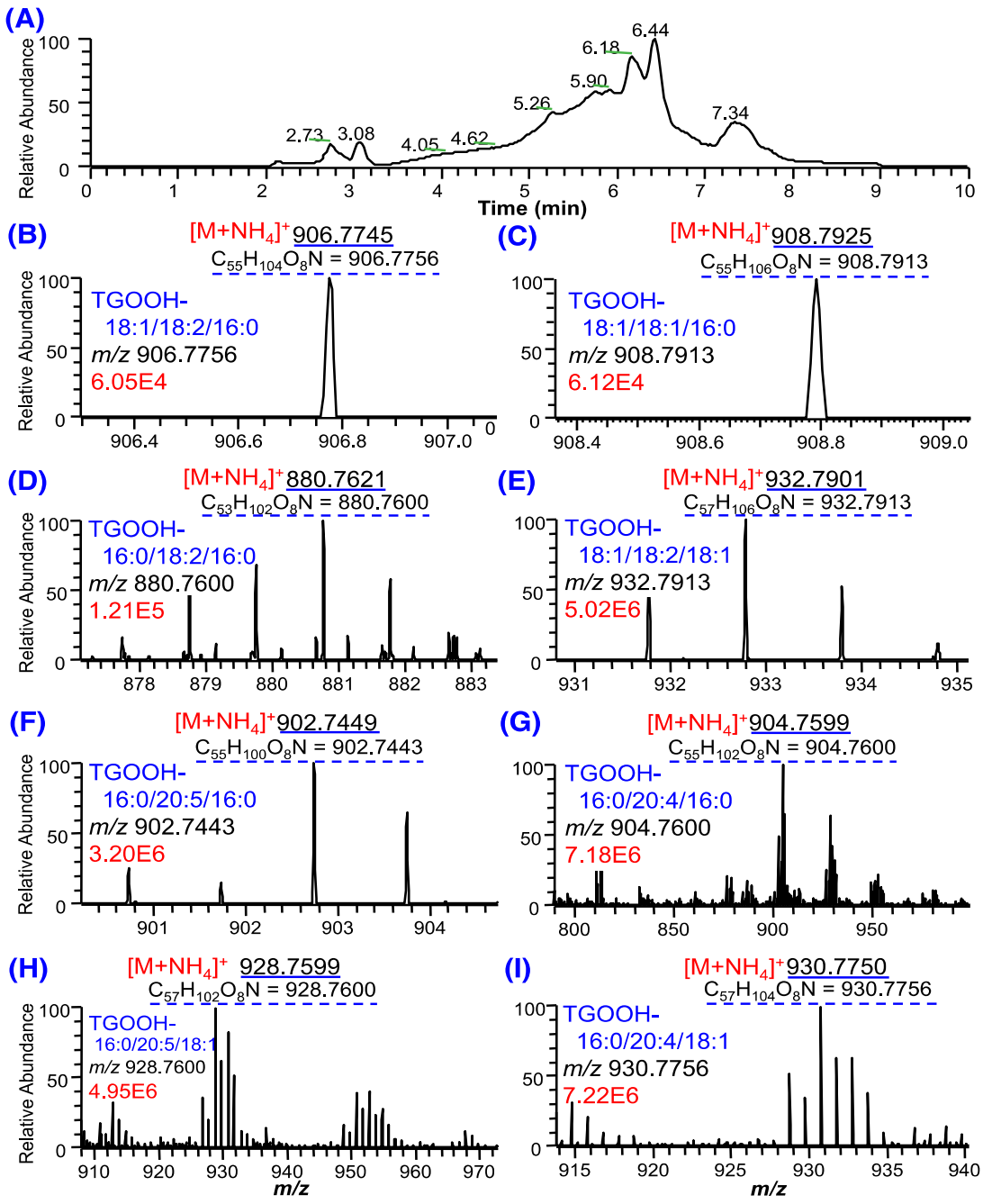

Figure 3.2 LC/LTQ Orbitrap profiles of triglyceride hydroperoxides (TGOOH) detected in freshly analyzed plasma in positive-ion mode: (A) total ion chromatogram of plasma extract; (B) mass spectrum showing *m/z* 906.7745 = TGOOH-18:1/18:2/16:0; (C) mass spectrum showing *m/z* 908.7925 = TGOOH-18:1/18:1/16:0; (D) mass spectrum showing *m/z* 880.7621 = TGOOH-16:0/18:2/16:0; (E) mass spectrum showing *m/z* 932.7901 = TGOOH-18:1/18:2/18:1; (F) mass spectrum showing *m/z* 902.7449 = TGOOH-16:0/20:5/16:0; (G) mass spectrum showing *m/z* 904.7599 = TGOOH-16:0/20:4/16:0; (H) mass spectrum showing *m/z* 928.7599 = TGOOH-16:0/20:5/18:1; (I) mass spectrum showing *m/z* 930.7750 = TGOOH-16:0/20:4/18:1.

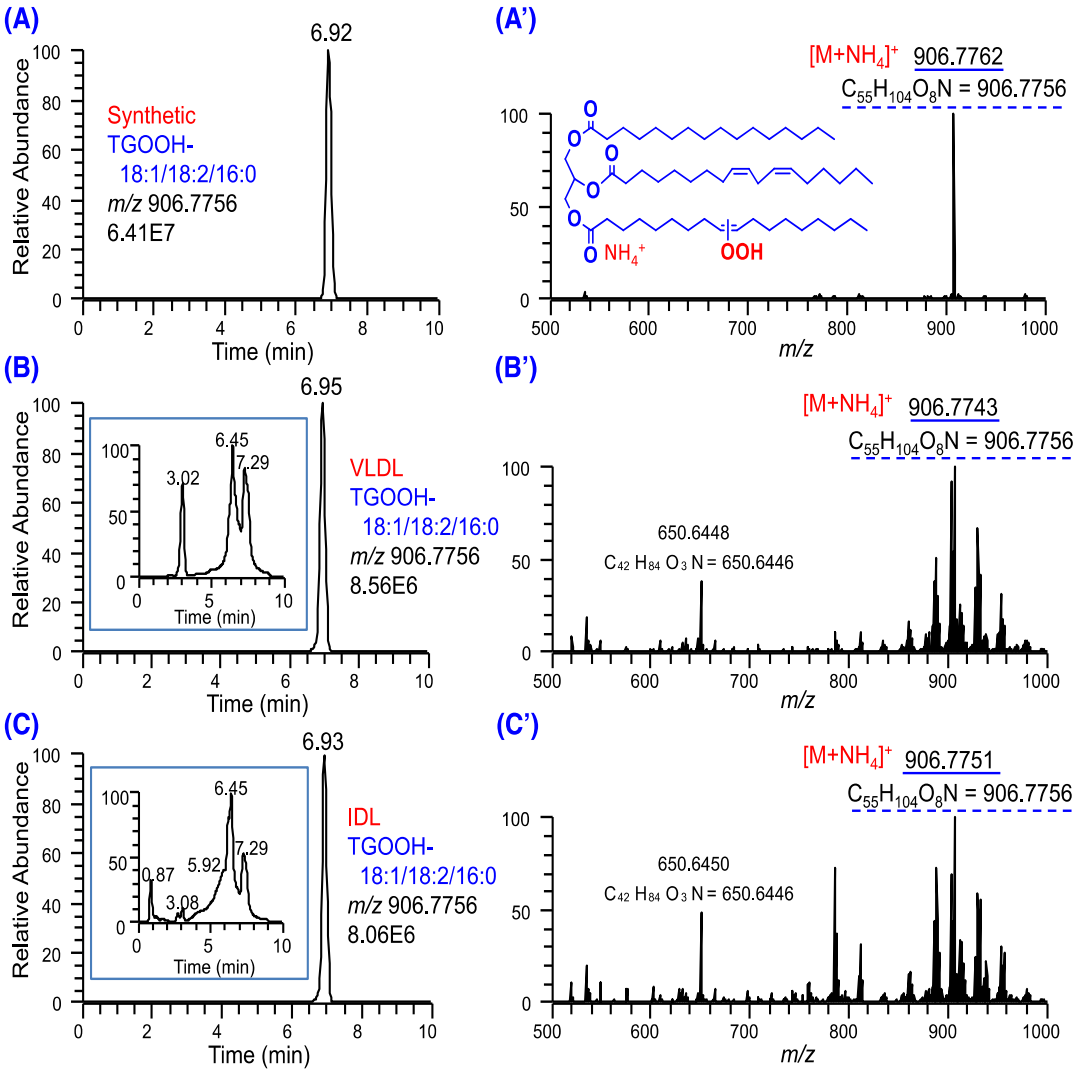

Figure 3.3 LC/LTQ Orbitrap profiles of TGOOH-18:1/18:2/16:0 in positive-ion mode: (A) extracted ion (*m/z* 906.7756) chromatogram of synthetic TGOOH-18:1/18:2/16:0 (40 pmol); (A') mass spectrum of peak associated with retention time of 6.92 min in (A); (B) total ion chromatogram (inside square) and extracted ion (*m/z* 906.7756) chromatogram of VLDL; (B') mass spectrum of peak associated with retention time at 6.95 min in (B) = TGOOH-18:1/18:2/16:0; (C) total ion chromatogram (inside square) and extracted ion (*m/z* 906.7756) chromatogram of IDL; (C') mass spectrum of peak associated with retention time at 6.93 min in (C) = TGOOH-18:1/18:2/16:0.

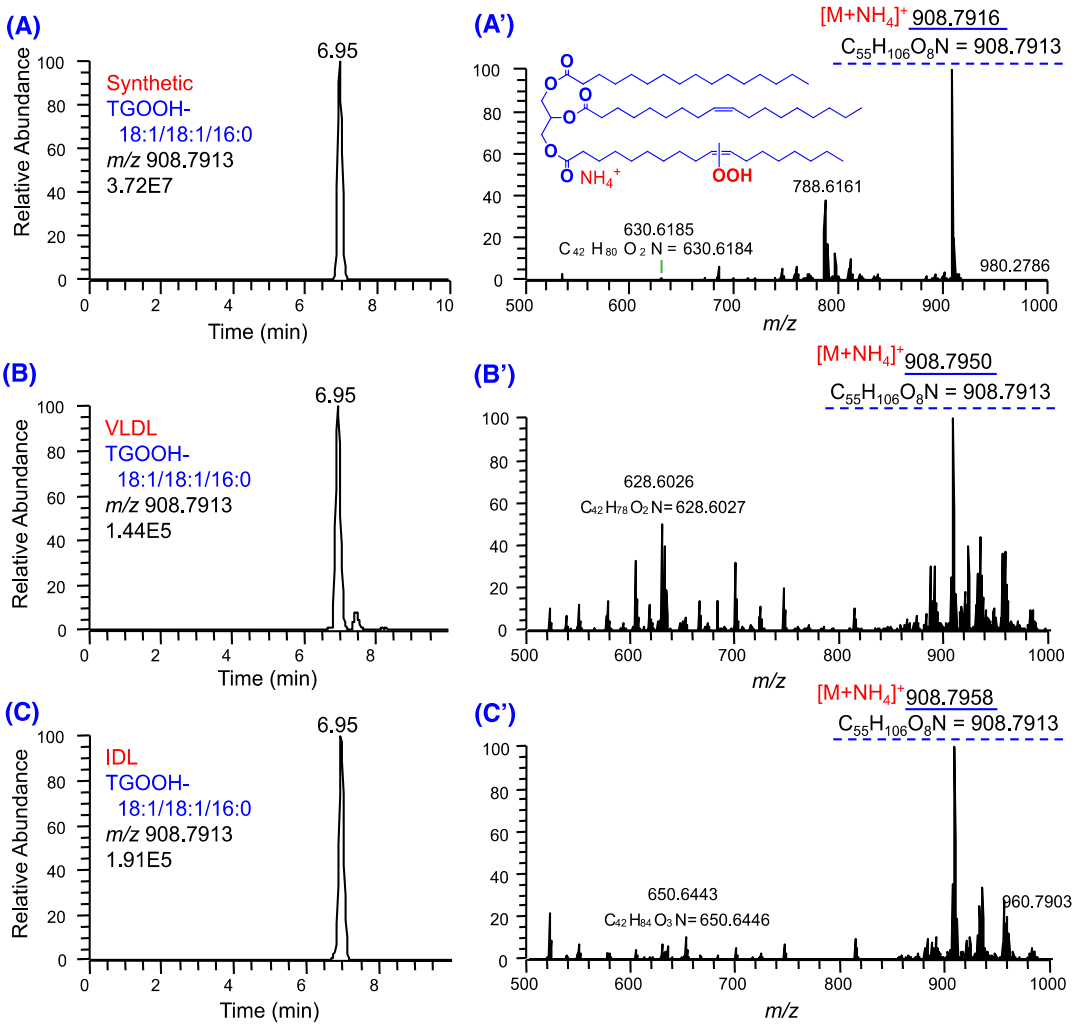

Figure 3.4 LC/LTQ Orbitrap profiles of TGOOH-18:1/18:1/16:0 in positive-ion mode: (A) extracted ion (*m/z* 908.7913) chromatogram of synthetic TGOOH-18:1/18:1/16:0 (40 pmol); (A') mass spectrum of peak associated with retention time of 6.95 min in (A); (B) extracted ion (*m/z* 908.7913) chromatogram of VLDL; (B') mass spectrum of peak associated with retention time at 6.95 min in (B) = TGOOH-18:1/18:1/16:0; (C) extracted ion (*m/z* 908.7913) chromatogram of IDL; (C') mass spectrum of peak associated with retention time at 6.95 min in (C) = TGOOH-18:1/18:1/16:0.

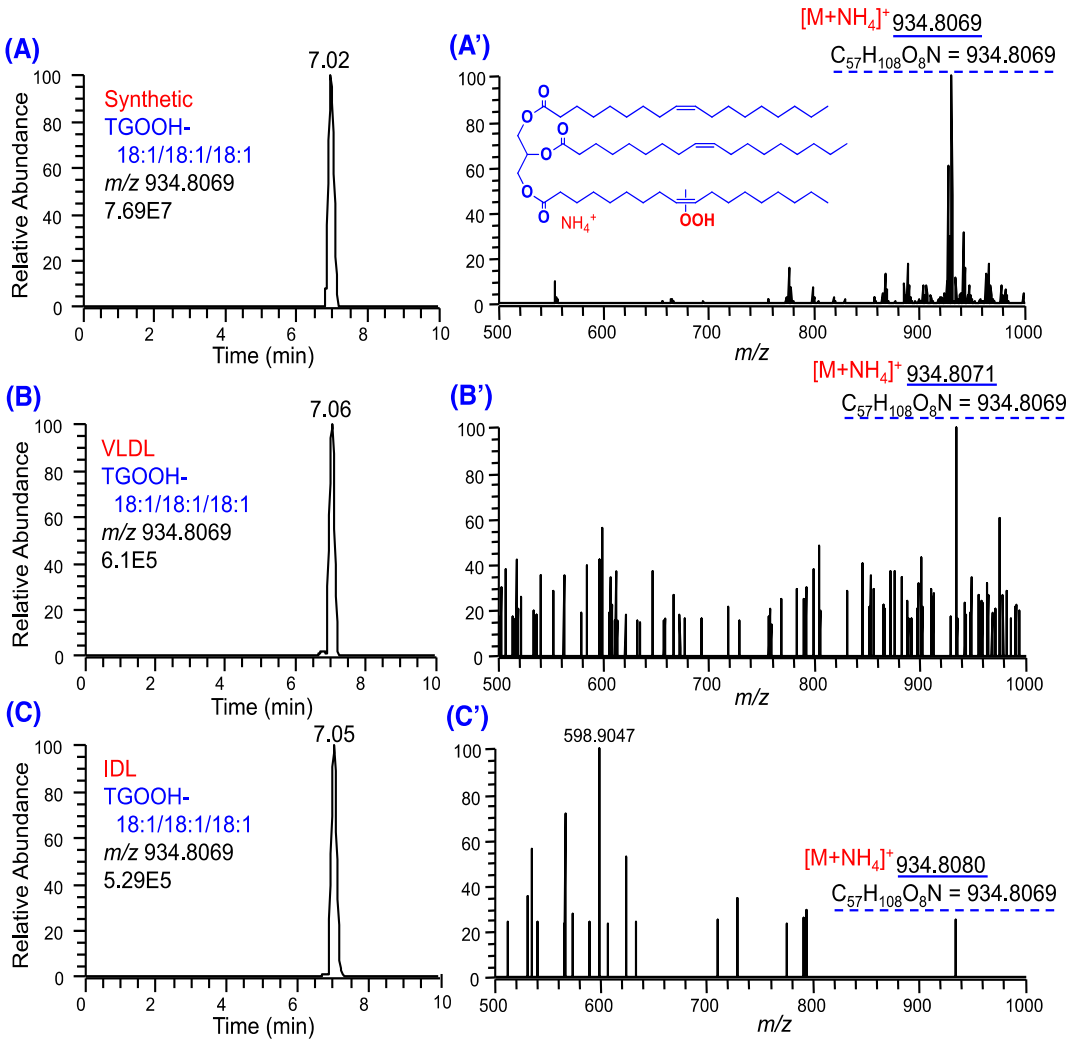

Figure 3.5 LC/LTQ Orbitrap profiles of triolein monohydroperoxide (TGOOH-18:1/18:1/18:1) in positive-ion mode: (A) extracted ion (*m/z* 934.8069) chromatogram of synthetic TGOOH-18:1/18:1/18:1 (40 pmol); (A') mass spectrum of peak associated with retention time of 7.02 min in (A); (B) extracted ion (*m/z* 934.8069) chromatogram of VLDL; (B') mass spectrum of peak associated with retention time at 7.06 min in (B) = TGOOH-18:1/18:1/18:1; (C) extracted ion (*m/z* 934.8069) chromatogram of IDL; (C') mass spectrum of peak associated with retention time at 7.05 min in (C) = TGOOH-18:1/18:1/18:1.

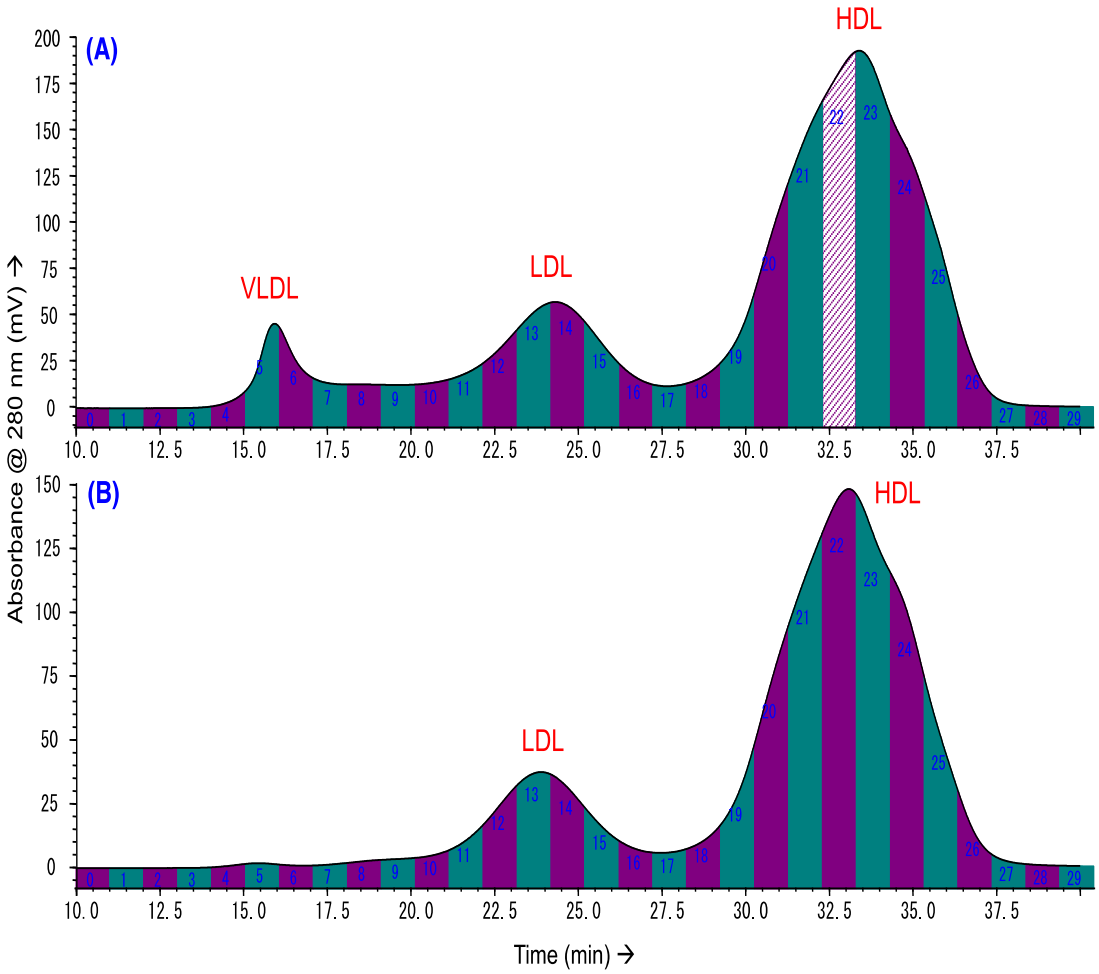

Figure 3.6 Size-exclusion HPLC chromatograms of lipoproteins from (A) total lipoprotein fraction (d<1.225), (B) lipoprotein fraction of *d*=1.019-1.225 isolated by ultracentrifugation.

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Chapter 4: Plasma Capric Acid Concentration in Healthy Subjects Determined by High-Performance Liquid Chromatography

4.1. Introduction

MCT are composed of MCFA, mainly octanoic acid and capric acid (decanoic acid, FA10:0). Unique metabolic properties made it lipid of interest to improve various clinical conditions and have been extensively reviewed.[1-6] Beside synthetic MCT, there are some natural source of MCFA. Coconut and palm kernel oils are rich in MCFA with more than 50 wt% of FA. MCFA are also present in milk from goat, cow, and human, for instance MCFA in bovine milk make up 4-12% of all FA.[7,8] Therefore, milk and milk products, especially butter are the important dietary source of FA10:0 in human nutrition. Measurement of plasma concentration of FA10:0 remains challenging due to its trace availability compared to LCFA in the systemic circulation, but can provide valuable information during therapeutic use of MCT.

Though gas chromatography (GC) is currently a routine procedure for FA analysis, it is associated with several disadvantages.[9] In particular, FA10:0 is relatively volatile than LCFA, and methyl derivatization adds more volatility to FA10:0, possibly resulting in low recovery during pre-analytical steps for GC. HPLC

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with appropriate derivatization overcomes this problem, further adding the advantages of speed, resolution, sensitivity and specificity. A wide variety of derivatization and detection systems are available including UV/visible, fluorescence, chemiluminescence, electrochemical, light-scattering, and mass spectrometry detection for the HPLC analysis of FA.[9,10] One of the simple and reliable HPLC approaches is derivatization of FA to its hydrazide using 2-nitrophenylhydrazine hydrochloride (2-NPH·HCl), followed by reversed phase separation and UV/visible detection.[11,12] Enzymatic methods for determination of FA have also been described.[13,14] Although a large number of techniques have been described for accurate and reproducible measurement of the FA in the plasma, none of them were designated specifically for FA10:0. Importantly, there is a paucity of statistical data on precision and accuracy in the measurement of plasma FA10:0.

This study describes (a) the development of a specific and sensitive HPLC method for the measurement of FA10:0 in the plasma, (b) analytical consideration for the measurement of FA10:0, and (c) plasma FA10:0 concentration in fasting and non-fasting healthy humans.

4.2. Materials and Methods

4.2.1. Chemicals

2-NPH·HCl, 1-(3- dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1-EDC·HCl), undecanoic (FA11:0), myristic (FA14:0), palmitic (FA16:0), heptadecanoic (margaric acid, FA17:0), and stearic acid (FA18:0) were purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). FA10:0 and pyridine were purchased from Wako Pure Chemical Industry (Osaka, Japan). Methanol and water

were of HPLC grade and from Wako Pure Chemical Industry (Osaka, Japan). FA labeling reagents were obtained from YMC CO. (Kyoto, Japan).

4.2.2. Synthesis of Standards

2-NPH-labeled FA10:0, FA14:0, FA16:0 and FA18:0 were used as standards while FA11:0 and FA17:0 were used as internal standards (IS). The solution of respective FA (FA10:0, FA11:0, FA14:0, FA16:0, FA17:0, and FA18:0; 5.0 mmol each), 2-NPH·HCL (5.5 mmol), 1-EDC·HCL (5.5 mmol), pyridine (3% v/v) in ethanol was heated at 60°C for 20 min to synthesize specific 2-NPH-labeled FA (FA-NPH). Formation of the products was monitored by thin layer chromatography (Silica gel 60 F_{254}, Merck Co., Darmstadt, Germany). The reaction products were purified by fractionation after thoroughly washing several times with HCl (5%), neutralized by $NaHCO_3$ (5%), distilled water, saturated solution of NaCl sequentially, and dried over Na_2SO_4, followed by the evaporation of the organic solvent using a rotary evaporator. Purification of the product by column chromatography on a silica gel 60N (100-120μm, Kanto Chemical Co., Inc., Tokyo, Japan) using chloroform-hexane (2:1, v/v) as an eluent and recrystallization of a homogeneous effluent from methanol gave pure FA-NPH as yellowish needle shaped crystals.

The melting points of the synthesized FA-NPH were measured by a micro melting point apparatus (Yanaco New Sciences Inc., Kyoto, Japan) and ranged from 94.1-94.8 (FA10:0-NPH), 96.5-97.3 (FA11:0-NPH), 103.8-104.7 (FA14:0-NPH), 107.5-108.0 (FA16:0-NPH), 109.4-109.8 (FA17:0-NPH), and 110.3-111.0°C (FA18:0-NPH). The purity of the standards was assessed by ^{1}H and ^{13}C-nuclear magnetic resonance (NMR) using JEOL JNM-AL400 (Tokyo, Japan) with $CDCl_3$ as solvent. ^{1}H-NMR ($CDCl_3$, 400 MHz) of the major rotamer of FA10:0-NPH, δ (ppm)

8.97 (*bs*, 1H, 8.16 (1H, dd, J = 1.4, 7.2 Hz, H-3), 7.47 (1H, dt, J = 1.4, 7.2 Hz, H-5), 7.04 (1H, dd, J = 0.9, 7.2 Hz, H-6), 6.86 (1H, dt, J =0.9, 7.2 Hz, H-4), 2.31 (*t*, *J* = 7.3 Hz, 2H, –CO–CH_2'–), 1.70 (2H, quin, J =7.2 Hz, H-3'), 1.21-1.35 (*m*, 12H, -CH_2'-), and 0.88 (*t*, 3H, J = 6.8 Hz, H-10'). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm) 172.94 ($C^{1'}$), 145.27 (C^{1}), 136.18 (C^{5}), 133.40 (C^{2}), 126.62 (C^{3}), 119.06 (C^{4}), 114.23 (C^{6}), 34.44 ($C^{2'}$), 31.99 ($C^{8'}$), 29.56, 29.45, 29.43, 29.39 ($C^{4'}$ to $C^{7'}$), 25.53 ($C^{3'}$), 22.80 ($C^{9'}$), 14.27 ($C^{10'}$). Molecular characterizations of synthesized FA-NPH were carried out by an ESI mass spectrometer (Thermo Scientific Finnegan LXQ, CA, USA). Base peaks in the mass spectra of FA10-NPH (M=307.2) are formed by ionization of the $[M+H]^+$ ion at *m/z* 308.2 and $[M-H]^-$ ion at *m/z* 306.3 on the positive and negative-ion mode, respectively (Figure 4.1).

4.2.3. Preparation of Standard Curve

A stock solution (200 μmol/L) of each FA-NPH was prepared gravimetrically in methanol by measuring its dry weight on an ultrasensitive electro-balance (Cubis® ultramicro balance, Sartorius, Goettingen, Germany). Working standards for HPLC analysis were prepared by mixing standard solutions of FA10:0-NPH, FA14:0-NPH, FA16:0-NPH and FA18:0-NPH to obtain the final concentration of each 0.1, 1.0, 2.0, 4.0, 6.0, 8.0, 20.0 μmol/L. Each standard solution also contained 1 and 4 μmol/L of IS FA11:0-NPH and FA17:0-NPH, respectively. The standard solutions were stored at -20°C and allowed to equilibrate at room temperature before use. Ten microliters of each standard solution was injected into the HPLC. The calibration curves were constructed by plotting the peak area ratio of each FA-NPH standard to the IS (FA11:0-NPH for FA10:0-NPH and FA14:0-NPH, and FA17:0-NPH for FA16:0-NPH and FA18:0-NPH) against its concentration.

4.2.4. Specimen

Fasting blood sample (3 mL) was collected from five healthy human volunteers (three male and two female; mean age ±SD, 31 ±9.3 years). In another 106 healthy Japanese volunteers (44 male and 62 female; mean age ±SD, 21.9 ±3.2 years), non-fasting blood samples (3 mL) were collected irrespective of dietary intake. Written informed consent was obtained from all study subjects. Plasma was separated from all of the samples within 30 min of collection and stored at -80°C until used. Ethics review boards of the Faculty of Health Sciences, Hokkaido University and of Oita University, Faculty of Medicine approved this study protocol.

4.2.5. Assay of FA in Plasma

For the derivatization of FA with 2-NPH, a method established by Miwa et al.[11], was used with the following modifications. For the FA10:0 assay, 50 μL of plasma was mixed with 10 μL of FA11:0 (IS, 2 nmol), while for FA16:0 and FA18:0 assay, 10 μL of plasma was mixed with 25 μL of FA17:0 (IS, 5 nmol). 100 μL of KOH (15 %, w/v in ethanol:water 50:50) was then added and the mixture was heated at 80°C for 20 min to ensure complete saponification of triglycerides present in the samples. This was followed by the addition of 200 μL of each 2-NPH·HCL (20 mM in ethanol) and 1-EDC·HCL (0.25 M in ethanol:pyridine 97:3) and heated at 60°C for another 20 min. After the addition of 200 μL of KOH (10%, w/v in methanol:water 50:50), the mixture was further heated at 60°C for 15 min and cooled. To the resulting mixture, 4 mL of potassium phosphate buffer (pH 4.6) and 3 mL of hexane was added. The mixture was then vortex mixed vigorously for 30 sec and centrifuged at 3000 × g for 30 min at 25°C. The upper hexane layer was collected and completely evaporated under vacuum (Tomy centrifugal concentrator, Tokyo, Japan). The residue was

dissolved in 200 μL of methanol and filtered using a centrifugal filter device (PVDF 0.1 μm, Merck Millipore Ltd., Carrigtwohill, Ireland). Ten microliters of the extract was injected into the HPLC.

4.2.6. Accuracy, Precision, Recovery and Stability

The recovery and reproducibility for the FA assay were investigated by repeating analysis of plasma of healthy human volunteers added with known concentrations of FA10:0, FA16:0 and FA18:0 at three different levels – low, normal and above-normal in the same run and on different days. Since FA10:0 is relatively soluble, pure FA10:0 was directly added in a fasting plasma (10.0 mL) that do not contain FA10:0, to obtain a final concentration of 200 μmol/L. The mixture was incubated at 37°C for an hour with mixing several times to ensure complete solubilization. The plasma containing FA10:0 was then diluted to 10, 50 and 160 μmol/L with plasma and 50 μL of each were used for measuring FA10:0 concentration. Additionally, aliquot of these plasma were also stored at -20°C and analyzed for FA10:0 on every successive month for six months to determine its stability. The solutions of FA16:0 and FA18:0 were added to 10 μL of plasma during the derivatization to give the final concentration of 155.2, 1055.52 and 2055.52 μmol/L of FA16:0 and 123.5, 1023.5 and 1823.5 μmol/L of FA18:0. Intra-assay and inter-assay precision were determined by analyzing FA in these spiked human plasma on different days (n=10) and on the same day (n=5) respectively.

4.2.7. Liquid Chromatography Condition

Chromatographic analyses were performed with a Shimadzu Prominence LC-20AD HPLC (Shimadzu Seisakusho, Kyoto, Japan) equipped with a Shimadzu Model CTO-

10A injector and the SPD-M20A photo diode array detector. The absorbance was measured at 400 nm. The separation was performed with C_4 Mightysil reversed-phase column [150 mm x 4.6 mm (i.d.); particle size 5μm] (Cica Reagent, Kanto chemical Co., Inc., Tokyo, Japan), maintained at 35°C. Gradient elution was performed with water [pH adjusted to 4.0 with 1% (v/v) trifluoroacetic acid] (solvent A) and methanol (solvent B), at a flow rate of 1.0 mL/min. The HPLC gradient conditions were as follows: 0.00-3.00 min isocratic at 60% B, 3.01-30.00 min 60→90% B, 30.01-35.00 min isocratic at 100% B to wash away any residual peaks eluting after last peak of interest and 35.01-40.00 min isocratic at 60% B to re-equilibrate the column.

4.3. Results

4.3.1. Separation of Fatty Acids in Plasma by HPLC

The separation of FA-NPH derivatives by reversed-phase HPLC is based on the chemical property of increasing polarity with decreasing chain length of FA. Therefore, by changing the polarity of solvent, the RT of FA can be adjusted. A series of experiments were performed to identify the optimal HPLC conditions, including types of columns and solvents, and its elution profiles, for the separation of four fatty acids in the plasma (FA10:0, FA14:0, FA16:0 and FA18:0) along with two IS (FA11:0 and FA17:0). The present HPLC method was selected as optimal for the distinct separation of FA without interference with other components in the plasma within elution time of 30 min. For the measurement of FA10:0, 15 min of run time is adequate as both FA10:0 and IS FA11:0 elute within the mentioned time. A typical chromatogram obtained from a mixture of standards and plasma samples were elucidated in Figure 4.2.

4.3.2. Calibration and Sensitivity

An eight-point calibration curve ranging from 1 to 200 pmol per injection for NPH derivatives of FA10:0, FA14:0, FA16:0 and FA18:0 were evaluated. When 10 μL of above-prepared calibration specimen was injected (0.1, 1.0, 2.0, 4.0, 6.0, 8.0, and 20.0 μmol/L) on the column, the response (y) was linearly related to FA concentration (x). Each calibration curve, as determined by linear regression analysis, showed good linearity with the correlation coefficient (r^2) of 0.99829, 0.99800, 0.99988, 0.99862 for FA10:0, FA14:0, FA16:0 and FA18:0, respectively (Figure 4.3).

4.3.3. Recovery and Reproducibility

The recovery and reproducibility of the present method were determined by analyzing the plasma of healthy volunteers added to three different concentrations of FA10:0, FA16:0 and FA18:0 for five times in a day (inter-day) and 10 different days (intra-day). The data of the inter-day and intra-day variations for the FA analysis are shown in Table 4.1. In-between run for FA10:0 added plasma to give concentrations of 10, 50 and 160 μmol/L, the assay values were 10.1 (SD 0.6, CV 5.4%), 51.5 (SD 2.6, CV 5.0%) and 152.3 (SD 1.9, CV 1.3%). With-in run for FA10:0 at identical concentrations, the assay values were 10.4 (SD 0.2, CV 2.3%), 51.3 (SD 0.8, CV 1.7%) and 162.9 (SD 6.3, CV 3.9%). The analytical recovery of FA10:0 ranged from 95.2 – 104.0%. The intra- and inter-assay CV were 1.9 - 3.6% for FA16:0, with the analytical recovery of 101.4 - 107.6%. FA18:0 assay showed relatively poor recovery of 82.9 - 94.9% with CV of 1.0 - 7.8%. The storage studies with added FA10:0 in human samples indicate no significant changes in its concentrations over a period of 6 months. Based on these data, this method is suitable for clinical uses to measure FA10:0 in the plasma.

4.3.4. FA10:0 in Human Plasma

No peak was detected at the elution time for FA10:0 in all fasting plasma of healthy humans (Figure 4.2C), indicating that the normal fasting plasma concentration of this FA is below the detection limit (0.1 μmol/L). Mean plasma concentration of total FA16:0 and FA18:0 in the fasting healthy volunteer (n=5) were 2092.1±244.1 and 696.8±76.3 μmol/L, respectively.

Mean plasma concentration of FA10:0 in the non-fasting blood of healthy volunteer (n=106) is 0.3 μmol/L (SD 0.4; Max 1.6). FA10:0 was not detected in 50 (47.2%) non-fasting blood samples, while 29 (27.4%) plasma contained FA10:0 less than or equal to 0.5 μmol/L (mean, 0.4; SD, 0.1) and 27 (25.5%) contained it at more than 0.5 μmol/L (mean, 0.9, SD, 0.3). We observed significant correlation of plasma FA10:0 concentration with FA14:0 (r = 0.278, p<0.05) but not with FA16:0 and FA18:0 (Table 4.2).

4.4. Discussion

The HPLC method for the measurement of FA10:0 described in this study is simple, accurate, and reproducible, which can be applied to monitor both free and total FA10:0 concentration during MCT therapy. The procedure is relatively non-expensive and rapid, as many samples can be processed in a single batch without undue effort and the reaction products are stable for many days at 4°C. Although the last peak of interest (FA18:0) elutes at about 30 min, it is necessary to run blank for a further 10 min, to provide sufficient time for elution of some unidentified peaks appearing after 30 min. However, for the measurement of FA10:0, 15 min of HPLC run is sufficient. In addition, several other factors contribute to the superiority of our method. First, we used FA11:0 as IS, which has similar physical and chemical

properties to FA10:0, in contrast to many reported methods using FA17:0, a LCFA as the IS. Second, high resolution and isolated peaks for these FA were observed in the chromatogram without interference. Third, the assays have very good linearity from 1 to 200 pmol per injection. Fourth, both total and free FA10:0 can be determined in the same HPLC condition and derivatization technique with and without saponification respectively. Lastly, the analytical recoveries were ranging from 95 to 104% with good reproducibility of the assay, making it suitable for the measurement of both free and total FA10:0 for clinical uses. Furthermore, this method can be also used to measure LCFA simultaneously. However, the recovery of FA18:0 was found to be lower than those of FA10:0 and FA16:0 (Table 4.1). The relatively low recovery of FA18:0 was also reported by Miwa H.[12] The low recoveries of FA18:0 might be attributed to a possibly less reactivity of FA18:0 with 2-NPH than those for shorter-chain fatty acids. When the labeling with 2-NPH is used for the measurement of short- to long-chain fatty acids, appropriate internal standards, according to the length of FA might be necessary.

FA10:0 was undetectable in the fasting plasma of healthy volunteers as consistent with other studies.[15,16] However, levels of FA16:0 and FA18:0 in these subjects were within the normal and acceptable ranges, which further add to the reliability of this method. FA10:0 concentration in the non-fasting samples were negligible. Only 27 (25.5%) of human volunteers have FA10:0 concentration above 0.5 μmol/L, with the maximum of 1.6 μmol/L, which is still more than 1000 times lower than the concentration of FA16:0, most predominant FA in human plasma. There is a scanty of data available about the plasma concentration of FA10:0. It is believed that majorities of dietary MCFA are carried to the liver through portal circulation and are completely metabolized within it. Therefore, the systemic

availability of FA10:0 is negligible unless MCT is used therapeutically. Existence of FA10:0 in trace amounts in some non-fasting individuals in this study may reflect dietary FA10:0 that escaped hepatic utilization. The most possible source of FA10:0 in the Japanese diet is milk and its products. Therefore, it appears that plasma level of FA10:0 depends on dietary status. Communities where coconuts and its oils are extensively taken orally, such as south India, particularly Kerala (Literally "Land of coconut palms"), possibly have higher plasma levels of FA10:0.

GC approach for measuring FA10:0 specifically have been reported.[15,17] Haidukewych et al. reported that the concentration of FA10:0 are below detection limit in plasma.[15] In contrast, Onkenhout et al. previously reported relatively higher concentration of plasma FA10:0 in control children using GC.[18] The higher level may be linked with higher consumption of milk in children compared to adults. HPLC based assay for the measurement of FA10:0 has been described by Dean et al.[19] They used α-p-dibromoacetophenone for the derivatization of MCFA and determined FA10:0 in plasma after treating children with MCT diet but not in healthy subjects. The derivatization of FA with NPH followed by UV-detection as described by Miwa et al. have been implicated for the determination of short-chain FA.[20,21] However, use of this labeling technique for the specific measurement of FA10:0 in plasma have not been reported yet. Our method can be particularly valuable to measure plasma FA10:0 during MCT therapy in order to acquire precise information about the therapeutic target of MCFA. As being readily digested by acid stable gastric lipase and predominantly absorbed via the portal vein, MCT have been historically used in the treatment of pancreatic insufficiency and fat malabsorption syndrome. In the recent years, the interest of dietary intervention with MCT is increasing to benefit varieties of clinical conditions including the obesity,[3,22,23] metabolic syndrome,[6] insulin

resistance,[24,25] liver diseases,[26] and cardiac diseases.[5] Furthermore, studies revealed that MCFA incorporates into the cellular lipids at a lower rate than LCFA and induce a lower accumulation of triglycerides.[27] This phenomenon is particularly advantageous in neutral lipids storage disorders. In addition, it appears that the mobilization of cytoplasmic MCT is independent to the action of adipose tissue triglyceride lipase (ATGL).[27] Therefore, it can be speculated that MCT therapy can reduce the lipid accumulation in ATGL mutated cells. It is of significance to monitor plasma free and total FA10:0 concentration, and its association with clinical signs and symptoms in patients receiving dietary therapy with MCT, which would provide scientific evidence about the mechanism that explains the benefit of MCT dietary therapy.

4.5. Conclusion

This study describes an improved method for the separation, derivatization and quantification of FA10:0 in plasma using HPLC with sufficient accuracy and reproducibility. FA10:0 is detected in trace amounts in healthy human during non-fasting state. Application of this method may be valuable in the monitoring of FA10:0 concentration during therapeutic use of MCT.

Tables and Figures

Table 4.1 Inter and intra-assay precision and accuracy of three fatty acids in serum evaluated by means of coefficients of variation and recovery of added standards from spiked sera (%).

		Intra-day (n=10)				Inter-day (n=5)			
Fatty Acid	Concentration [Expected] (µmol/L)	Concentration [Observed] Mean±SD (µmol/L)	Precision [CV] (%)	Recovery (% of expected value)	Recovery range (%)	Concentration [Observed] Mean±SD (µmol/L)	Precision [CV] (%)	Recovery (% of expected value)	Recovery range (%)
Decanoic acid (FA10:0)	10.0	10.1±0.6	5.4	100.3	89.1-108.0	10.4±0.2	2.3	104.0	101.0-106.0
	50.0	51.5±2.6	5.0	102.9	97.5-105.4	51.3±0.8	1.7	102.5	101.0-105.4
	160.0	152.3±1.9	1.3	95.2	94.1-97.6	162.9±6.3	3.9	101.8	95.6-106.3
Palmitic acid (FA16:0)	155.5	157.7±3.0	1.9	101.4	99.6-104.3	157.7±5.7	3.6	102.2	97.8-106.9
	1055.5	1167.8±41.9	3.6	110.6	104.2-110.9	1104.8±38.7	3.5	104.6	100.4-109.57
	2055.5	2212.1±65.0	2.9	107.6	105.1-113.0	2138.9±64.9	3.0	104.1	100.6-107.7
Stearic acid (FA18:0)	123.5	102.4±7.4	7.3	82.9	77.0-91.3	116.4±4.0	3.4	94.3	91.1-98.4
	1023.5	854.1±8.7	1.0	83.5	82.6-84.7	954.6±49.3	5.2	93.3	90.3-99.6
	1823.5	1590.5±31.4	2.0	87.2	84.7-89.2	1726.4±134.4	7.8	94.9	85.0-101.6

Table 4.2 Spearman's correlation between plasma saturated fatty acids concentration (n=106)

	FA10:0 (μmol/L)	FA14:0 (μmol/L)	FA16:0 (μmol/L)	FA18:0 (μmol/L)
FA10:0 (μmol/L)	1.000	0.278†	0.022	0.006
FA14:0 (μmol/L)	0.278†	1.000	0.597†	0.436†
FA16:0 (μmol/L)	0.022	0.597†	1.000	0.727†
FA18:0 (μmol/L)	0.006	0.436†	0.727†	1.000

† Correlation is significant at $p < 0.01$

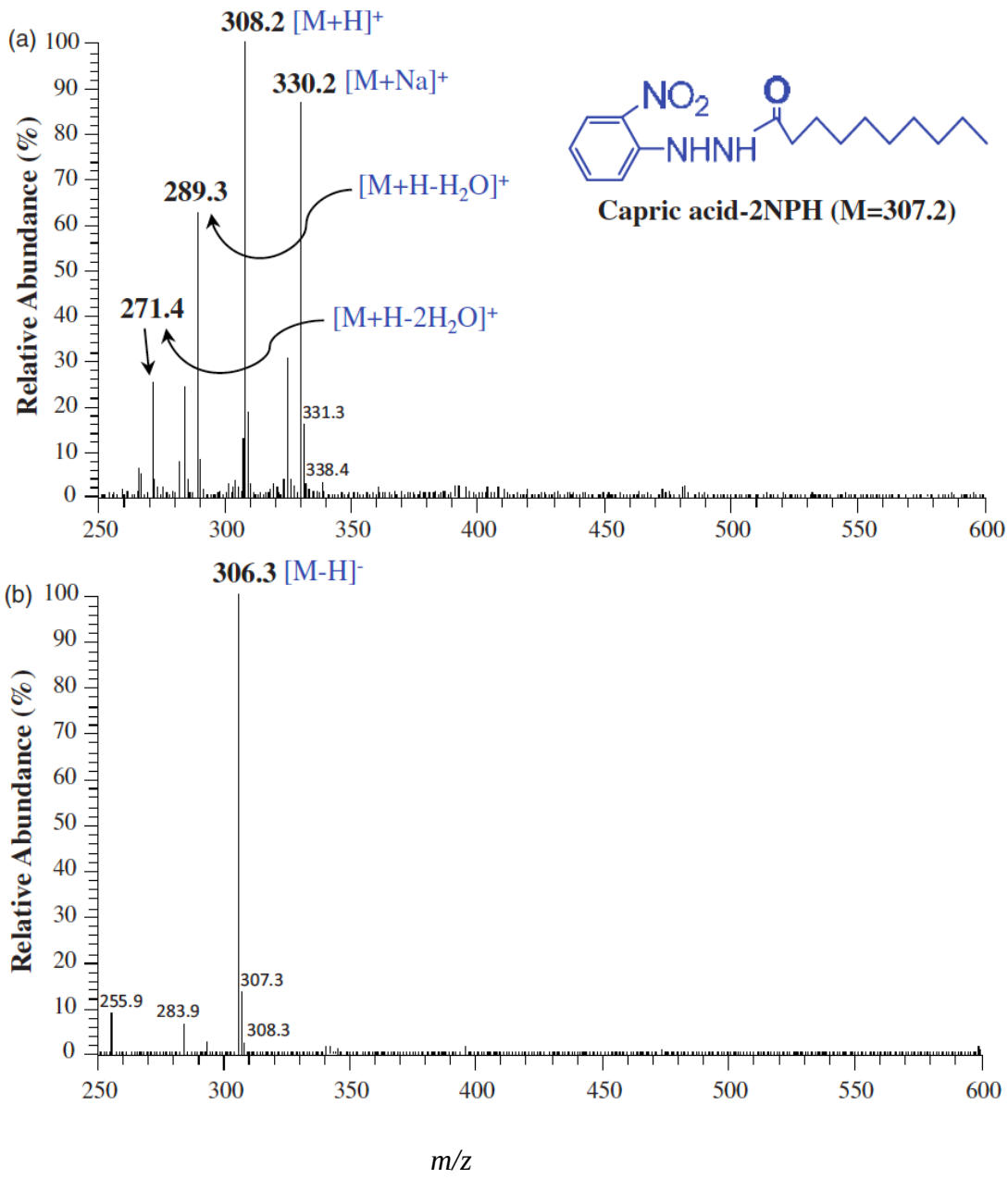

Figure 4.1 Electrospray ionization mass spectra of NPH-derivative of capric acid in (a) positive- and (b) negative-ion mode. Key diagnostic ion peaks are annotated in bold type with their *m/z* value.

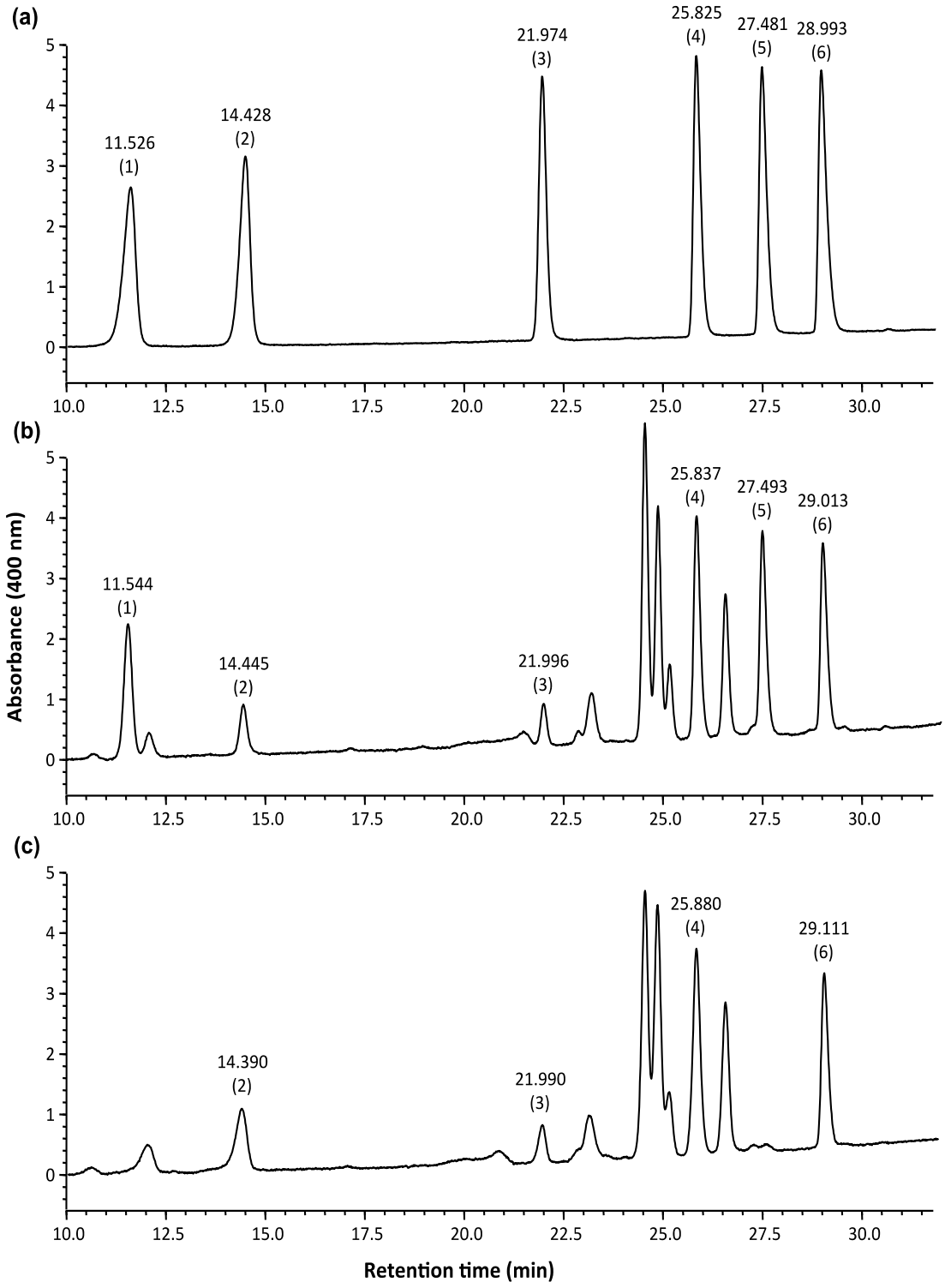

Figure 4.2. HPLC chromatogram of the 2-nitrophenylhydrazine derivatives of (a) mixture of standard fatty acids, (b) fatty acid from a human plasma spiked with FA10:0 (200 μmol/L) and (c) fatty acids from a fasting human plasma. Peaks: 1, capric (FA10:0); 2, undecanoic (FA11:0); 3, myristic (FA14:0); 4, palmitic (FA16:0); 5, heptadecanoic (FA17:0); and 6, stearic acid (FA18:0).

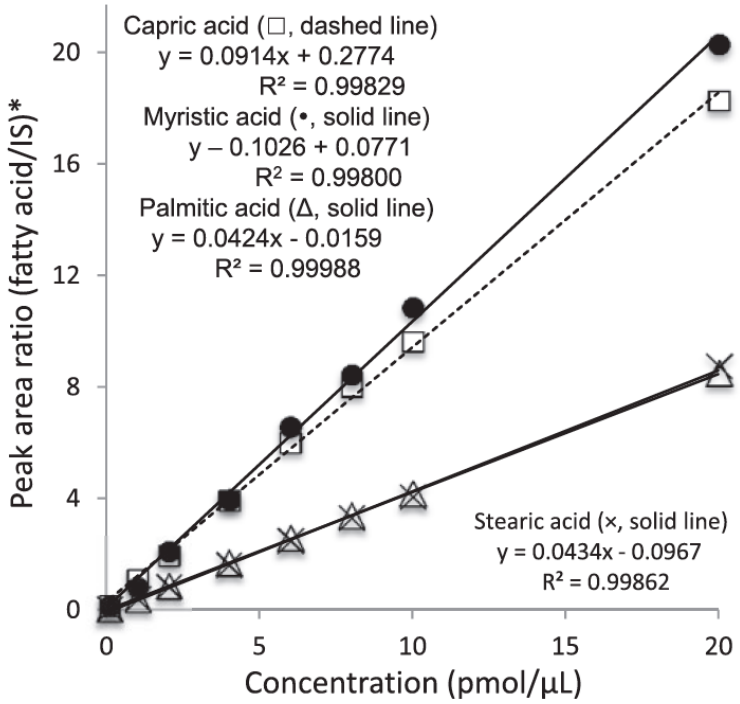

Figure 4.3 Calibration curves for 2-nitrophenylhydrazine derivatives of capric (FA10:0), myristic (FA14:0), palmitic (FA16:0) and stearic acid (FA18:0).

**undecanoic acid (FA11:0) was used as IS for capric (FA10:0) and myristic acid (FA14:0), and heptadecanoic acid (FA17:0) was used as IS for palmitic (FA16:0) and stearic acid (FA18:0).*

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Chapter 5: Summary and Perspectives

These studies were conducted to investigate analytical considerations in the measurement of some emerging lipids, namely CEOOH, TGOOH and FA10:0 that may significantly influence human health and nutrition. Because cholesterol is considered as the main culprit in the development of CHD, oxidation of cholesterol will definitely add to its atherogenicity. Therefore, it is important to analyze CEOOH in the plasma and lipoproteins, which would provide valuable information about atherogenesis and serve as a potentially useful biomarker for the identification of individuals who are at high risk for future coronary events. Similarly, the existence of oxidized triglycerides may provide new insight into understanding the pathogenicity of TRL. Therefore, this study was focused on qualitative and quantitative analysis of TGOOH in the plasma and its distribution in lipoproteins. Likewise, MCFA have special value in human nutrition because they are potentially useful to improve clinical outcomes in various disease conditions, including TGCV. In light of this importance, we developed a reliable assay for the measurement of FA10:0, which would be useful in the therapeutic monitoring of MCT dietary therapy. There are several remarkable findings of this study, which are summarized as follows:

First, as discussed in the chapter 2, this study confirmed that the CEOOH detected in the plasma were carried in VLDL and IDL. Total lipids were extracted from the plasma, VLDL, and IDL fractions of six healthy donors. Highly sensitive LC/LTQ Orbitrap mass spectrometric analysis of lipid extracts of VLDL and IDL demonstrated six molecular species of CEOOH, namely Ch18:1-OOH, Ch18:2-OOH,

Ch18:3-OOH, Ch20:4-OOH, Ch20:5-OOH, and Ch22:6-OOH, on the basis of their mass spectra and RT on the LC. Except for Ch18:3-OOH, all CEOOH species were also identified in the plasma samples. Despite being relatively poor in cholesterol, both VLDL and IDL contained detectable amounts of CEOOH. Furthermore, although VLDL and IDL can induce foam cell formation *in vitro*, the role of these TRL in atherosclerosis is poorly understood. The detection of CEOOH molecules in the TRL might indicate a possible involvement of these hydroperoxides in the atherogenicity of TRL.

Second, in addition to the existence of CEOOH in VLDL and IDL, these TRL also contained TGOOH. This is the first study to elucidate the presence of oxidized triglycerides in human plasma and lipoproteins in their native form. Ten molecular species of TGOOH were found in VLDL and IDL. TGOOH-16:0/18:2/16:0, TGOOH-18:1/18:2/16:0, TGOOH-18:1/18:1/18:1, TGOOH-18:1/18:2/18:1, TGOOH-16:0/20:4/16:0, TGOOH-16:0/20:5/18:1, TGOOH-16:0/20:4/18:1 and TGOOH-16:0/22:6/18:1 were detected in all VLDL and IDL samples. Although mean percentage content of triglycerides in IDL is almost half of VLDL, the mean concentration of TGOOH is much higher in IDL compared to that of VLDL (512±173.2 vs 349.8±253.6 pmol per μmol of triglycerides). In contrast, LDL and HDL fractions were devoid of any TGOOH. Similarly, this study identified and detected ten molecular species of TGOOH in the plasma, though only TGOOH-18:1/18:1/16:0 and TGOOH-16:0/18:2/16:0 were present in all nine plasma samples. The mean concentration of plasma TGOOH was 56.1±25.6 pmol per μmol of triglycerides. This study confirmed that TGOOH detected in the plasma were carried by TRL but not other lipoproteins. It is likely that the hydrolysis of triglycerides present in these TRL, during the course of metabolism, will release significant

amounts of oxidized FA, which, in turn, can elicit inflammatory responses in the endothelial wall. Therefore, the presence of TGOOH in the TRL is possibly associated with its atherogenicity; demonstration of oxidized triglycerides in VLDL and IDL may provide new insight into understanding the pathogenicity of TRL.

Third, this study reported an improved HPLC method designed specifically to measure FA10:0 in the plasma. Briefly, FA10:0 in the samples were labeled with 2-nitrophenylhydrazine, followed by extraction and quantification using an UV-visible detector in HPLC. FA11:0, which has similar physical and chemical properties to FA10:0, was used as an internal standard. The results of this study indicate that the present method is simple, rapid, accurate, sensitive, and reproducible. The lower detection limit of this assay is 0.1 μmol/L. The inter-assay coefficient of variation of this assay at concentrations of 10, 50, and 160 μmol/L were 5.4% (Mean±SD, 10.1±0.6), 5.0% (Mean±SD, 51.5±2.6), and 1.3% (Mean±SD, 152.3±1.9), respectively, and the intra-assay coefficient of variation at the identical concentrations were 2.3% (Mean±SD, 10.4±0.2), 1.7% (Mean±SD, 51.3±0.8), and 3.9% (Mean±SD, 162.9±6.3), respectively. The analytical recovery of FA10:0 ranged from 95.2 – 104.0%. This method was applied to determine FA10:0 concentration in the fasting plasma of five healthy volunteers (male/female, 3/2; mean age, 31±9.3 years) and non-fasting plasma of 106 volunteers (male/female, 44/62; mean age, 21.9±3.2 years). The plasma concentrations of FA10:0 in the healthy individuals were negligible. All of the fasting plasma (n=5) and 47.2% (n=50) of non-fasting blood samples lacked detectable amounts of FA10:0, while 27.4% (n=29) of the non-fasting plasma sample contained FA10:0 less than or equal to 0.5 μmol/L (Mean±SD, 0.4±0.1), and 25.5% (n=27) contained FA10:0 at more than 0.5 (up to 1.6 μmol/L) (Mean±SD, 0.9±0.3). It is generally believed that majority of dietary MCFA are carried out to the liver

through portal circulation and get completely metabolized within it. Therefore, the systemic availability of FA10:0 is negligible. The existence of trace amounts of FA10:0 in some non-fasting individuals in this study may reflect dietary FA10:0 that have escaped hepatic utilization.

The findings of this study have several noteworthy implications that can be applied to existing and future studies. This study demonstrated that TRL contains oxidized cholesterol and triglycerides; these oxidized lipids may be involved in the atherogenicity of the TRL. Further works are needed to explore the association of CEOOH and TGOOH in the atherosclerotic process. The molecular species of CEOOH and TGOOH detected in this study should become the targets of future quantification studies. The author believes that the present study forms a foundation to select appropriate target molecules and internal standards for the measurement of oxidized lipids and determination of their clinical utility. Additionally, the HPLC method presented in this study can be applied to monitor the MCFA concentrations during therapeutic use of MCT. The author believes that such assay will provide scientific evidence about the mechanism that explains the benefit of the MCT therapy, and aid in determining appropriate dose and frequency of such therapy.

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List of original publications, upon which this dissertation is based

1. **Shrestha R**, Hui SP, Miura Y, Yagi A, Yuji T, Seiji T, Fuda H, and Chiba H. Identification of molecular species of oxidized triglyceride in plasma and its distribution in lipoprotein. *Clin Chem Lab Med* 2015 (In-press, DOI: 10.1515/cclm-2014-1088).
2. **Shrestha** R, Hui SP, Imai H, Hashimoto S, Uemura N, Takeda S, Fuda H, Suzuki A, Yamaguchi S, Hirano K, and Chiba H. Plasma capric acid concentration in healthy subjects determined by UV-labeled HPLC. *Ann Clin Biochem* 2015 (In-press, DOI: 10.1177/0004563215569081).
3. **Shrestha R**, Hui SP, Sakurai T, Yagi A, Takahashi Y, Takeda S, Jin S, Fuda H, and Chiba H. Identification of molecular species of cholesteryl ester hydroperoxides in very low-density and intermediate-density lipoproteins. *Ann Clin Biochem* 2014; 51:662-71.

List of other publications

1. Takeda S, Subagyo A, Hui SP, Fuda H, **Shrestha R**, Sueoka K, Chiba H. Elastic modulus of LDL as potential indicator of its oxidation. *Ann Clin Biochem* 2015 (In-press, DOI: 10.1177/0004563215584958).
2. Sigdel M, Kumar A, Gyawali P, **Shrestha R**, Tuladhar ET, and Jha B. Association of high sensitivity C-reactive protein with the components of metabolic syndrome in diabetic and non-diabetic individuals. *J Clin Diagn Res* 2014; 8:CC11-3.
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7. Poudel B, Mittal A, **Shrestha R**, Nepal AK, and Shukla PS. Prostate biomarkers with reference to body mass index and duration of prostate cancer. *Asian Pac J Cancer Prev* 2012; 13:2149-52.
8. **Shrestha R**, Gyawali P, Yadav BK, Dahal S, Poudel B, Khanal M, and Jha B, Sapkota B. In-vitro assessment of cell-mediated immunity by demonstrating effector-T cells for diagnosis of tuberculosis in Nepalese subjects. *Nepal Med Coll J* 2011; 13:275-8.
9. B Rijal, **R Shrestha**, and B Jha. Association of thyroid dysfunction among infertile women visiting infertility center of Om Hospital, Kathmandu, Nepal. *Nepal Med Coll J* 2011; 13:247-9.
10. **Shrestha R**, Jha SC, Khanal M, Gyawali P, Yadav BK, and Jha B. Association of cardiovascular risk factors in hypertensive subjects with metabolic syndrome defined by three different definitions. *JNMA J Nepal Med Assoc* 2011; 51:157-63.
11. Yadav BK, Gupta RK, Gyawali P, **Shrestha R**, Poudel B, Sigdel M, and Jha B. Effects of long-term use of depo-medroxyprogesterone acetate on lipid metabolism in Nepalese women. *Korean J Lab Med* 2011;31:95-7.
12. **Shrestha R**, Gyawali P. High sensitivity C-reactive protein: emerging biomarker for primary prevention of cardiovascular disease. JNMA *J Nepal Med Assoc* 2010: 49(179):263 [Letter]
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14. Lakhey M, Ghimire R, **Shrestha R**, and Bhatta AD. Correlation of serum free prostate-specific antigen level with histological findings in patients with prostatic disease. *Kathmandu Univ Med J (KUMJ).* 2010;8:158-63.

15. **Shrestha R**, Sigdel M, Poudel B, Gyawali P, Shrestha M, Yadav BK, Sharma V, Khanal M, and Jha B. Assessment of urinary VMA level in suspected padriatic cases of catecholamines producing neurochromaffin tumors**.** *J Nepal Asso Med Lab Sci* 2009;10:23-5.
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18. **Shrestha R**, Gyawali P, Poudel B, Sigdel M, Khanal M. In response to the article entiltled "Prevalence of difference type of gallstone in the patients with cholelithiasis at Kathmandu medical college. *Kath Uni Med J (KUMJ)* 2009:7(4);477-8. [Letter]
19. Yadav BK, Chhetri GB, Poudel B, Sigdel M, Gyawali P, Regmi P, and **Shrestha R**. Serum uric acid level in obese and non-obese individuals. *J Nepal Asso Med Lab Sci* 2009;10:27-30.
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23. Mishra SK, Sah JP, **Shrestha R**, and Lakhey M. Emergence of nalidixic acid resistant Salmonella: A confounding scene in antibiotic armamentarium. *J Nepal Asso Med Lab Sci* 2008;9:61-64.

List of presentations

[Oral]

1. *"Quantification of plasma capric acid using high-performance liquid chromatography"*, at 3rd International symposium on Triglyceride deposit cardiomyovasculopathy and Neutral lipid storage disorders, Tokyo, Japan. [March 14, 2015]
2. "*Analysis of free and total plasma capric acid after therapeutic administration of medium-chain triglycerides in patients with triglyceride deposit cardiomyovasculopathy"* at 24th annual meetings of Japanese Society of Clinical Chemistry- Hokkaido, Sapporo, Japan. [Oct 25, 2014]
3. "*Identification of Medium-chain triglycerides in fibroblast from adipose triglyceride lipase deficiency by LC/MS*", at 23rd annual meeting of Japanese Society of Clinical Chemistry- Hokkaido. Sapporo, Japan. [Dec 7, 2013]
4. "*Qualitative analysis of cholesteryl ester hydroperoxides in plasma, VLDL and IDL by LC/MS*", at the 60th National congress of Japanese Society of Laboratory Medicine, Kobe, Japan. [Oct 31-Nov 3, 2013]
5. "*Qualitative analysis of triacylglycerol hydroperoxide in VLDL and IDL by LC/MS*", at the 53rd Annual meeting of Japanese Society of Clinical Chemistry, Tokushima, Japan. [Aug 30-Sept 1, 2013]
6. *"Principle of External Quality control: Principle and practice, Laboratory documents and Post-analytical consideration"* at a symposium organized by Bangladesh Association of Medical Biochemist, Dhaka, Bangladesh. [Jan 7-8, 2012] (Invited)
7. *"Metabolic Syndrome: an update, discrepancy in prevalence with different criteria, association with emerging risk factors and laboratory testing"* at Metabolic Syndrome and Nutrition Symposium, organized by Kathmandu University Hospital & Embassy of Israel, Dhulikhel, Nepal. [May 11, 2011] (Invited)
8. *"High-sensitivity C-reactive protein as a promising marker in the risk stratification of coronary heart disease in hypertensive subjects"* BANCON 2011, National Academy of Sciences and Technology, Lalitpur, Nepal. [April 8-9, 2011]

9. "*In-vitro assessment of cell mediated immunity for diagnosis of tuberculosis*" on conference organized by Association of Clinical Pathology Nepal (ACPN), Kathmandu, Nepal. [Aug 15, 2009]
10. A talk on *"Use of Control Chart in Laboratory Quality Control"* on Workshop entitled Workshop on Total Quality Management in Laboratory Medicine. Tribhuvan University Teaching Hospital, Kathmandu, Nepal. [July 10, 2009] (Invited)
11. A talk on *"Interpretation of Control Chart: A multirole Concepts"* on Workshop entitled Workshop on Total Quality Management in Laboratory Medicine. Tribhuvan University Teaching Hospital, Kathmandu, Nepal. [July 11, 2009] (Invited)
12. *"Evaluation of mycobacteriophage assay for rapid diagnosis of tuberculosis"* organized by Nepal Assocaition for Medical Laboratory Sciences, Kathmandu, Nepal. [June 20, 2009]
13. A talk on *"Renal Function Test in Laboratory Medicine"* on CME program entitled Role of Clinical Chemistry Laboratory in Medicine. Tribhuvan University Teaching Hospital, Kathmandu, Nepal. [April 4, 2009] (Invited)

[Poster]

1. "*Analysis of Lipid hydroperoxides in triglyceride-rich lipoproteins*", at the 38th annual meeting of the Japanese Society for Biomedical Mass Spectrometry, Kobe, Japan. [Sept 26-27, 2013]
2. "*Qualitative determination of triacylglycerol hydroperoxide in VLDL, Intermediate-density lipoprotein and human plasma using Orbitrap mass spectrometer*", at the annual meeting of American Association for Clinical Chemistry 2013, Houston, Texas, USA [July 28–Aug 1, 2013]
3. "*Detection of Medium-chain triglyceride in fibroblast from adipose triglyceride lipase deficiency by Orbitrap*", at the 2nd international symposium on Triglyceride Deposit Cardiomyovasculopathy and Neutral Lipid Storage Disease", Osaka, Japan. [April 19-20, 2013]
4. *"In-vitro Assessment of Cell-Mediated Immunity by Demonstrating Effector-T Cells for Diagnosis of Tuberculosis and its Cross Reactivity to Leprosy in*

Nepalese Subjects" at annual meeting of American Association for Clinical Chemistry 2011, Atlanta, Georgia, USA. [July 24–28, 2011]

5. *"Serum lipids and anti-oxidized LDL antibody in Nepalese preeclamptic subjects"* at annual meeting of American Association for Clinical Chemistry 2011, Atlanta, Georgia, USA. [July 24–28, 2011]
6. *"Protein:Creatinine Ratio in Random Urines Accurately Predict 24 Hour Protein Loss in Nepalese Patients with Chronic Kidney Disease"* at International Federation of Clinical Chemistry and Laboratory Medicine (IFCC)- WorldLab- EuroMedLab, Berlin, Germany. [May 15-19, 2011]
7. *"Serum lipids, high sensitivity CRP, Anti-oxidized LDL antibody and Urine albumin in Nepalese subjects with hypertension, diabetes and both"* at International Federation of Clinical Chemistry and Laboratory Medicine (IFCC)-WorldLab- EuroMedLab, Berlin, Germany. [May 15-19, 2011]
8. *"Association of Cardiovascular Risk Factors in Hypertensive Subjects with Metabolic Syndrome Defined by Three Different Definitions"* on Asia Pacific Congress of Clinical Biochemistry 2010, Seoul, South Korea. [Oct 3-7, 2010]
9. *"Association of high sensitivity C-reactive protein and anti-oxLDL antibody in Nepalese hypertensive subject"* at annual meeting of American Association for Clinical Chemistry 2010, Anaheim, California, USA. [July 24-29, 2010]
10. "*Association of high sensitivity C-reactive protein and anti-oxLDL antibody in Nepalese hypertensive subject*" on student poster contest of American Association for Clinical Chemistry 2010, Anaheim, California, USA. [July 26, 2010]

Awards:

1. Selected for "Best Publication Award 2014" for article entitled "Identification of molecular species of cholesteryl ester hydroperoxides in very low-density and intermediate-density lipoproteins. *Ann Clin Biochem* 2014; 51:662-71" by Japanese Society of Clinical Chemistry (JSCC).
2. National Academy of Clinical Biochemistry (NACB) Distinguished Abstract Award 2013, USA.
3. International Travel Grant Award 2011 by International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) and Asian-pacific Federation of Clinical Biochemsitry (APFCB) for IFCC-Euro LabMed congress, Berlin, Germany.
4. American Association for Clinical Chemistry (AACC)'s "Van Slyke Foundation 5 Years Membership Grant Award (2011–2016).
5. International Travel Grant Award 2010 by Asian-pacific Federation of Clinical Biochemistry (APFCB) for APCCB, Seoul, South Korea.
6. International Travel Grant Award 2010 by American Association for Clinical Chemistry (AACC).