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Short communication

Application of enzymatic fluorometric assays to quantify phosphatidylcholine, phosphatidylethanolamine and sphingomyelin in human plasma lipoproteins

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ABSTRACT

Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SM) are important surface components of plasma lipoproteins, including very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). However, the pathophysiological roles of PC, PE and SM in lipoproteins have not been well characterized owing to the difficulties in quantifying phospholipid classes in lipoproteins. In this study, we assessed the precision and accuracy of the enzymatic fluorometric assays for measuring PC, PE and SM in VLDL, LDL and HDL, which were isolated from human plasma by ultracentrifugation. The within-run coefficients of variation (CV) for the measurements of PC, PE and SM in lipoproteins were 1.5–2.8 %, 1.1–2.4 % and 0.9–2.3 %, respectively, whereas the between-run CVs for the PC, PE and SM assays were 2.7–4.7 %, 2.1–4.5 % and 1.6–3.3 %, respectively. Excellent linearity and almost complete recovery were achieved for all assays measuring PC, PE and SM in VLDL, LDL and HDL. Our preliminary results using these enzymatic fluorometric assays suggested that the phospholipid compositions were different among VLDL, LDL and HDL. In conclusion, we established high-throughput enzymatic fluorometric assays to quantify PC, PE and SM in human plasma VLDL, LDL and HDL, which will be useful for further investigation of pathophysiological roles of phospholipids in lipoproteins.

1. Introduction

Phospholipids are essential components of plasma lipoproteins including high-density lipoproteins (HDL), low-density lipoproteins (LDL), very-low-density lipoproteins (VLDL) and chylomicrons. Phospholipids, together with cholesterol, are necessary to constitute a lipoprotein-surface monolayer, which envelops a hydrophobic core consisting of triglyceride and cholesteryl esters (Jonas and Phillips, 2008; Morita, 2016). Phosphatidylcholine (PC) is the primary phospholipid component of lipoproteins, and sphingomyelin (SM) is the second most abundant phospholipid in lipoproteins (Jonas and Phillips, 2008; Morita, 2016). The lipoprotein-surface phospholipids play key roles in the lipoprotein metabolism by controlling the binding of apolipoproteins and enzymes to lipoproteins. For example, apolipoprotein E bound on the lipoprotein remnant surface increases the uptake of remnants into hepatocytes via the LDL receptor, LDL receptor-related protein and heparan sulfate proteoglycans (Mahley and Huang, 2007; Morita, 2016; Morita et al., 2003; Morita et al., 2011; Sakurai et al., 2005; Wakita et al., 2015). Apolipoprotein C-II is essential for the activation of lipoprotein lipase at the surface of VLDL, whereas apolipoprotein C-III strongly inhibits the lipoprotein lipase-mediated lipolysis and promotes hepatic VLDL assembly and secretion (Morita, 2016; Wolska et al., 2017; Yamamoto et al., 2003; Zheng, 2014).

It is well known that high levels of LDL-cholesterol and low levels of HDL-cholesterol increase the risk of coronary artery disease (Morita, 2016). In addition, it has been reported that plasma SM levels and SM/(PC + SM) ratios are positively and independently correlated with coronary artery disease (Jiang et al., 2000). PC, SM and phosphatidylethanolamine (PE) are incorporated into VLDL particles in hepatocytes and then secreted into the circulation. As SM, but not PC, is resistant to

Abbreviations: HDL, High-density lipoproteins; LDL, Low-density lipoproteins; VLDL, Very-low-density lipoproteins; PC, Phosphatidylcholine; POPC, 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; PE, Phosphatidylethanolamine; POPE, 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; SM, Sphingomyelin; SMase, Sphingomyelinase; Amplex Red, 10-Acetyl-3,7-dihydroxyphenoxazine; CV, Coefficients of variation.

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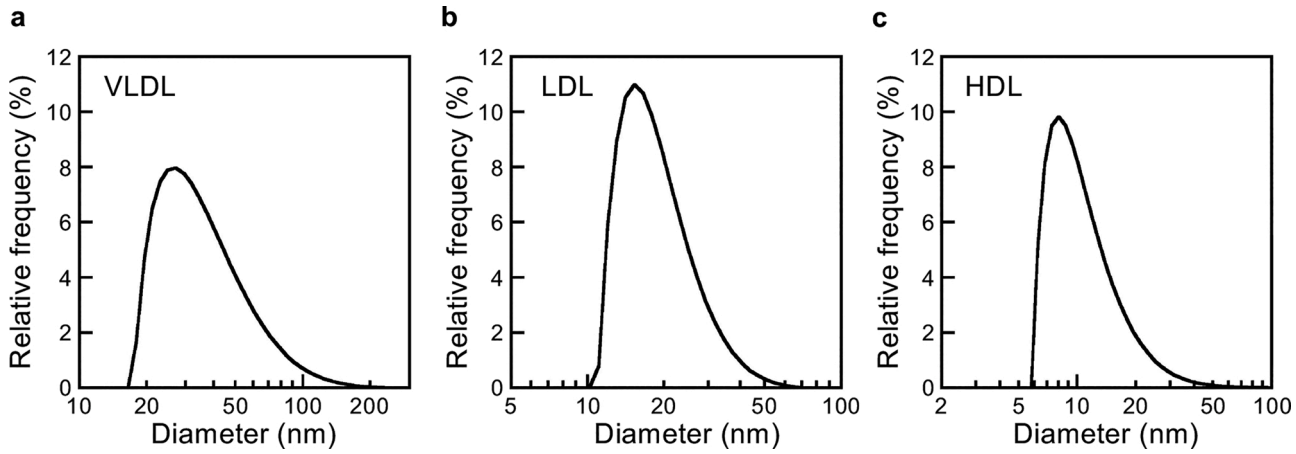

Fig. 1. Size distribution of lipoprotein particles. Human plasma VLDL (**a**), LDL (**b**) and HDL (**c**) fractions were isolated by the ultracentrifugation method. The volume-weighted distribution of particle sizes was assessed by dynamic light scattering. The volume-weighted mean diameters of VLDL, LDL and HDL were 39.1, 19.6 and 11.7 nm, respectively.

Table 1
Within-run reproducibility of measurements of PC, PE and SM in plasma VLDL, LDL and HDL.

Sample	Mean ± S.D. (nmol/mL plasma)	CV (%)	Mean ± S.D. (nmol/mL plasma)	CV (%)	Mean ± S.D. (nmol/mL plasma)	CV (%)
	PC in VLDL		**PC in LDL**		**PC in HDL**	
A-1	162.4 ± 2.6	1.6	1001.6 ± 20.0	2.0	503.5 ± 14.1	2.8
B-1	112.1 ± 2.1	1.9	546.7 ± 8.4	1.5	411.8 ± 7.5	1.8
C-1	249.8 ± 5.4	2.1	784.2 ± 20.2	2.6	443.3 ± 7.2	1.6
	PE in VLDL		**PE in LDL**		**PE in HDL**	
A-2	5.2 ± 0.1	2.4	46.6 ± 0.6	1.3	41.6 ± 0.8	2.0
B-2	12.1 ± 0.2	1.6	34.0 ± 0.5	1.4	24.6 ± 0.4	1.7
C-2	16.4 ± 0.2	1.1	25.9 ± 0.4	1.6	15.9 ± 2.2	1.6
	SM in VLDL		**SM in LDL**		**SM in HDL**	
A-3	20.8 ± 0.5	2.2	294.0 ± 5.8	2.0	85.0 ± 1.1	1.3
B-3	14.0 ± 0.3	2.0	165.5 ± 2.6	1.5	69.5 ± 1.0	1.4
C-3	18.9 ± 0.2	0.9	225.5 ± 5.2	2.3	77.2 ± 0.9	1.2

Samples for PC, PE and SM measurements were isolated from different plasma samples. Ten aliquots of each sample were simultaneously measured by the enzymatic fluorometric assays (n = 10).

Table 2
Between-run reproducibility of measurements of PC, PE and SM in plasma VLDL, LDL and HDL.

Sample	Mean ± S.D. (nmol/mL plasma)	CV (%)	Mean ± S.D. (nmol/mL plasma)	CV (%)	Mean ± S.D. (nmol/mL plasma)	CV (%)
	PC in VLDL		**PC in LDL**		**PC in HDL**	
A-1	155.2 ± 6.1	3.9	957.3 ± 45.4	4.7	506.3 ± 17.3	3.4
B-1	110.9 ± 4.5	4.0	556.2 ± 18.8	3.4	411.8 ± 12.6	3.1
C-1	241.0 ± 6.9	2.8	800.4 ± 16.2	2.0	435.5 ± 11.6	2.7
	PE in VLDL		**PE in LDL**		**PE in HDL**	
A-2	5.9 ± 0.3	4.3	50.9 ± 2.1	4.0	47.6 ± 1.3	2.8
B-2	13.2 ± 0.3	2.2	36.7 ± 0.8	2.1	26.4 ± 0.8	3.1
C-2	17.8 ± 0.8	4.4	27.5 ± 1.0	3.8	17.1 ± 0.8	4.5
	SM in VLDL		**SM in LDL**		**SM in HDL**	
A-3	20.2 ± 0.5	2.2	299.2 ± 7.9	2.6	82.6 ± 2.7	3.3
B-3	15.1 ± 0.5	3.2	181.9 ± 5.4	3.0	72.4 ± 1.1	1.6
C-3	20.4 ± 0.6	2.7	247.7 ± 6.7	2.7	81.1 ± 2.5	3.1

Samples for PC, PE and SM measurements were isolated from different plasma samples. Each sample was measured in duplicate once daily for 10 days by the enzymatic fluorometric assays with the same reagent lots (n = 10). To avoid repeated freeze-thaw cycles, the sample solution was separated into ten sample tubes and frozen at −20 °C immediately after preparation.

hydrolysis by lipoprotein lipase, hepatic lipase or lecithin:cholesterol acyltransferase, the SM/PC ratio in LDL is higher than that in VLDL (Morita, 2016). We have previously demonstrated that SM suppresses the binding of apolipoprotein E to the particle surface and the apolipoprotein E-mediated cellular uptake of the particles (Morita et al., 2003). Sphingomyelinase (SMase) hydrolyzes SM to ceramide and phosphorylcholine (Morita et al., 2004). The treatment of LDL with SMase leads to the aggregation and fusion of the particles (Schissel et al., 1996). We have also observed ceramide-enriched microdomains in the SMase-treated particles by confocal microscopy (Morita et al., 2005). Alteration of LDL by SMase promotes the internalization of LDL into macrophages and foam cell formation (Marathe et al., 2000). Thus, the generation of ceramide from SM contained in LDL is considered to be an important step in atherogenesis. Dashti et al. have observed abundant PE in VLDL by high-performance thin layer chromatography analysis (Dashti et al., 2011). During lipolysis of VLDL, PE may be more preferably hydrolyzed than PC (Zhao et al., 2009). The clearance of VLDL with a high ratio of PE/PC is promoted (Zhao et al., 2009). However, little is known about whether alteration of the amount of PC, PE and SM in each lipoprotein fraction, VLDL, LDL or HDL, correlates with the onset or progression of diseases.

Enzymatic assays are simple, rapid and high-throughput. The specific enzymatic measurements of PC, PE and SM in serum or plasma have been performed and validated (Hidaka et al., 2008; Hojjati and Jiang, 2006; Hokazono et al., 2011). On the other hand, the accuracy of enzymatic measurements of PC, PE and SM has not been confirmed for isolated lipoprotein fractions. We have developed fluorometric assays for all major phospholipid classes, including PC, PE and SM, using combinations of specific enzymes and 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red), which enable high-throughput and high-sensitive quantification in pico-molar ranges (Morita et al., 2012a; Morita et al., 2012b; Morita et al., 2010; Morita and Terada, 2015; Morita et al., 2020; Morita et al., 2009; Tsuji et al., 2019). Recently, we have revealed that the phospholipid class compositions of HepG2 cells and their intracellular organelles are altered during cell growth using these enzymatic fluorometric assays (Tsuji et al., 2021). In the present study, we applied these enzymatic fluorometric assays to quantify PC, PE and SM in human plasma VLDL, LDL and HDL, and assessed their precision and accuracy.

2. Material and methods

2.1. Materials

Triton X-100 was obtained from Roche Diagnostics (Mannheim, Germany). Amplex Red Reagent and Amplex Red Stop Regent were

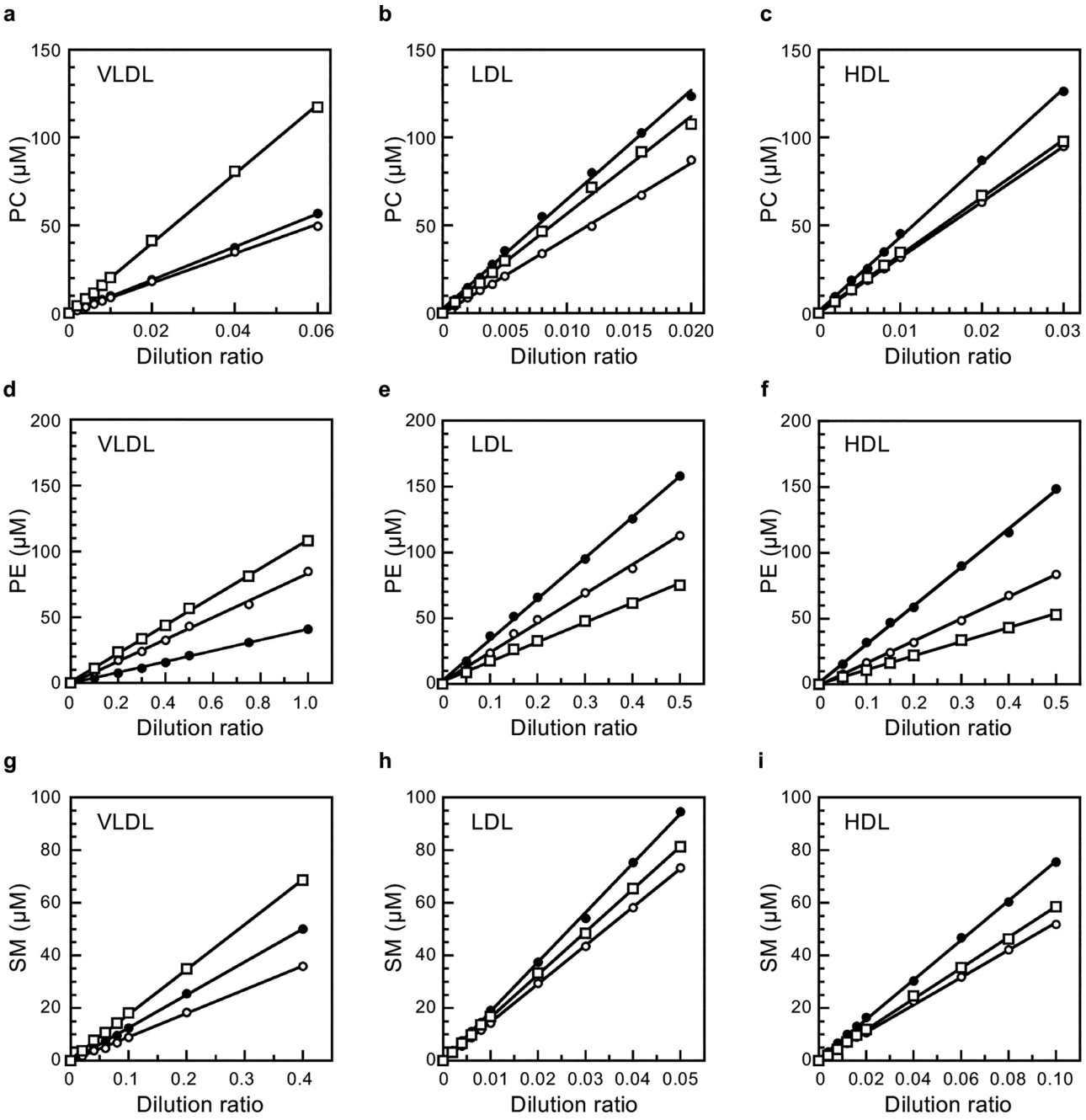

Fig. 2. Linearity of measurements of PC, PE and SM in VLDL, LDL and HDL. **S**amples for PC, PE and SM measurements were isolated from different plasma samples collected from three different subjects (subject *A*, closed circles; subject *B*, open circles; subject *C*, open squares). The lipid extracts from VLDL, LDL and HDL were sequentially diluted in 1% Triton X-100. PC concentrations in VLDL (**a**), LDL (**b**) and HDL (**c**) (sample *A*-1, *B*-1 and *C*-1), PE concentrations in VLDL (**d**), LDL (**e**) and HDL (**f**) (sample *A*-2, *B*-2 and *C*-2), and SM concentrations in VLDL (**g**), LDL (**h**) and HDL (**i**) (sample *A*-3, *B*-3 and *C*-3) were measured by the enzymatic fluorometric assays. (**a**) Sample *A*-1, $r = 0.9999$; sample *B*-1, $r = 0.9991$; sample *C*-1, $r = 0.9997$. (**b**) Sample *A*-1, $r = 0.9988$; sample *B*-1, $r = 0.9995$; sample *C*-1, $r = 0.9982$. (**c**) Sample *A*-1, $r = 0.9996$; sample *B*-1, $r = 1.000$; sample *C*-1, $r = 0.9997$. (**d**) Sample *A*-2, $r = 0.9995$; sample *B*-2, $r = 0.9989$; sample *C*-2, $r = 0.9996$. (**e**) Sample *A*-2, $r = 0.9994$; sample *B*-2, $r = 0.9985$; sample *C*-2, $r = 0.9986$. (**f**) Sample *A*-2, $r = 0.9995$; sample *B*-2, $r = 0.9997$; sample *C*-2, $r = 0.9995$. (**g**) Sample *A*-3, $r = 0.9998$; sample *B*-3, $r = 0.9996$; sample *C*-3, $r = 0.9999$. (**h**) Sample *A*-3, $r = 0.9997$; sample *B*-3, $r = 1.000$; sample *C*-3, $r = 0.9999$. (**i**) Sample *A*-3, $r = 0.9998$; sample *B*-3, $r = 0.9995$; sample *C*-3, $r = 0.9997$.

purchased from Thermo Fisher Scientific (Waltham, MA, USA). 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and SM from chicken egg (egg SM) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Phospholipase D from *Streptomyces chromofuscus* and tyramine oxidase from *Arthrobacter sp*. were obtained from Asahi Kasei Pharma (Tokyo, Japan). Peroxidase from horseradish roots was purchased from Oriental Yeast (Tokyo, Japan). All other chemicals used were of the highest reagent grade.

2.2. Subjects and blood sampling

Blood samples from healthy volunteers (three males aged 32–49 years, subjects *A*, *B* and *C*) after 12-h overnight fasting were collected in sterilized vacuum tubes containing EDTA-2Na (Venoject II, Terumo, Tokyo, Japan) using 23-gauge needles. The collected blood samples

Table 3
Recovery of PC added to lipid extract from plasma VLDL, LDL and HDL.

Sample	Added PC (μM)	Measured PC (μM)	Expected PC (μM)	Recovery (%)
VLDL				
A	0	53.5	–	–
	20	73.1	73.5	99.5
	40	93.1	93.5	99.6
	60	110.5	113.5	97.4
B	0	43.3	–	–
	20	62.8	63.3	99.2
	40	83.5	83.3	100.2
	60	102.5	103.3	99.2
C	0	47.9	–	–
	20	67.5	67.9	99.4
	40	89.5	87.9	101.8
	60	108.0	107.9	100.1
LDL				
A	0	37.7	–	–
	20	57.4	57.7	99.5
	40	78.5	77.7	101.0
	60	97.0	97.7	99.3
B	0	20.4	–	–
	20	39.0	40.4	96.5
	40	61.0	60.4	101.0
	60	82.3	80.4	102.4
C	0	29.8	–	–
	20	47.4	49.8	95.2
	40	69.2	69.8	99.1
	60	87.4	89.8	97.3
HDL				
A	0	26.3	–	–
	20	46.9	46.3	101.3
	40	65.8	66.3	99.2
	60	85.3	86.3	98.8
B	0	16.7	–	–
	20	35.1	36.7	95.6
	40	55.9	56.7	98.6
	60	77.2	76.7	100.7
C	0	17.2	–	–
	20	36.1	37.2	97.0
	40	56.3	57.2	98.4
	60	76.5	77.2	99.1

POPC standard solution was added to the lipid extract. The concentration of PC was measured by the enzymatic fluorometric assay.

Table 4
Recovery of PE added to lipid extract from plasma VLDL, LDL and HDL.

Sample	Added PE (μM)	Measured PE (μM)	Expected PE (μM)	Recovery (%)
VLDL				
A	0	10.8	–	–
	20	31.0	30.8	100.6
	40	52.3	50.8	103.0
	60	72.1	70.8	101.8
B	0	23.0	–	–
	20	43.2	43.0	100.5
	40	64.5	63.0	102.4
	60	84.3	83.0	101.6
C	0	32.1	–	–
	20	51.6	52.1	99.0
	40	72.0	72.1	99.9
	60	90.2	92.1	97.9
LDL				
A	0	40.4	–	–
	20	60.1	60.4	99.5
	40	79.4	80.4	98.8
	60	99.0	100.4	98.6
B	0	29.8	–	–
	20	49.1	49.8	98.6
	40	69.5	69.8	99.6
	60	91.9	89.8	102.3
C	0	22.6	–	–
	20	41.3	42.6	96.9
	40	62.5	62.6	99.8
	60	81.6	82.6	98.8
HDL				
A	0	34.4	–	–
	20	54.6	54.4	100.4
	40	73.4	74.4	98.7
	60	94.5	94.4	100.1
B	0	18.4	–	–
	20	37.6	38.4	97.9
	40	58.7	58.4	100.5
	60	79.1	78.4	100.9
C	0	12.9	–	–
	20	32.8	32.9	99.7
	40	54.0	52.9	102.1
	60	74.2	72.9	101.8

POPE standard solution was added to the lipid extract. The concentration of PE was measured by the enzymatic fluorometric assay.

were placed on ice and centrifuged (1200 *g*, 10 min, 4 °C) within 30 min of collection to obtain blood plasma samples. This study was approved by the ethics committee of Shiga University of Medical Science (R2019−242), and all subjects provided written informed consent. Our study was conducted in accordance with the Declaration of Helsinki.

2.3. Plasma lipoprotein isolation

Immediately after plasma collection, VLDL, LDL and HDL fractions were isolated by ultracentrifugation using a himac CS120FNX ultracentrifuge with a fixed angle rotor S110AT (Eppendorf Himac Technologies, Hitachinaka, Japan) according to the previously described method with slight modification (Chunta et al., 2016; Chunta et al., 2018). Briefly, 1.0 mL of solution A (0.195 M NaCl; density 1.006 *g*/mL) was layered on the top of 2.0 mL of plasma in a 3.4-mL polycarbonate tube. After centrifugation (650,000 *g*, 90 min, 4 °C), the VLDL fraction (1.0 mL) was collected from the top layer. Next, 1.0 mL of solution B (0.195 M NaCl and 2.44 M NaBr; density 1.182 *g*/mL) was added to the bottom layer containing LDL, HDL and other plasma proteins. After mixing and centrifugation (650,000 *g*, 130 min, 4 °C), the LDL fraction (1.0 mL) was collected from the top layer. Then, 1.0 mL of solution C (0.195 M NaCl and 7.65 M NaBr; density 1.478 *g*/mL) was added to the bottom layer and mixed. Centrifugation (650,000 *g*, 210 min, 4 °C) yielded the HDL fraction in the top layer (0.4 mL). Particle sizes of the isolated lipoproteins were measured by dynamic light scattering using Litesizer 100 (Anton Paar, Graz, Austria).

2.4. Phospholipid extraction

Phospholipids were extracted from the isolated plasma lipoprotein fractions by the method of Folch (Folch et al., 1957; Tsuji et al., 2019; Wakelam et al., 2007). In brief, sample solution/chloroform/methanol (3:8:4) was vortexed, incubated overnight at 4 °C and centrifuged to complete phase separation. After removal of the upper aqueous phase and the interfacial material, the recovered lower organic phase was washed with H_2O, and the aqueous phase was removed again. The organic solvent was evaporated from the lower phase. The evaporated sample was dissolved by adding 1% Triton X-100 (250 μL) and filtered through Durapore hydrophilic polyvinylidene fluoride membrane (0.45-μm pore size, Ultrafree-MC, Merck-Millipore, Billerica, MA, USA). We have confirmed that PC, PE and SM were almost completely recovered by the Folch method (Tsuji et al., 2021).

2.5. Measurement of phospholipid contents in human plasma lipoproteins

The PC and SM contents in the extracts of lipoproteins were quantified by enzymatic fluorometric methods (Morita et al., 2012a; Morita et al., 2010). Before the enzymatic fluorometric assay, a sample containing a high concentration of PC, PE or SM was diluted to be within the measurable range. POPC or egg SM dissolved in 1% Triton X-100 solution was used as the standard for measuring PC and SM, respectively. For PE measurement, we slightly modified the enzymatic fluorometric method that we have previously reported (Morita et al., 2010). Reagent

Table 5
Recovery of SM added to lipid extract from plasma VLDL, LDL and HDL.

Sample	Added SM (μM)	Measured SM (μM)	Expected (μM)	Recovery (%)
VLDL				
A	0	19.8	–	–
	20	39.4	39.8	99.0
	40	60.3	59.8	100.8
	60	80.6	79.8	101.0
B	0	27.1	–	–
	20	45.8	47.1	97.2
	40	68.6	67.1	102.2
	60	87.1	87.1	100.0
C	0	41.0	–	–
	20	61.0	61.0	100.0
	40	80.4	81.0	99.3
	60	99.7	101.0	98.7
LDL				
A	0	22.6	–	–
	20	41.5	42.6	97.4
	40	63.0	62.6	100.6
	60	83.8	82.6	101.5
B	0	15.4	–	–
	20	35.2	35.4	99.4
	40	56.5	55.4	102.0
	60	77.1	75.4	102.3
C	0	16.8	–	–
	20	36.3	36.8	98.6
	40	57.8	56.8	101.8
	60	75.2	76.8	97.9
HDL				
A	0	21.7	–	–
	20	40.5	41.7	97.1
	40	61.7	61.7	100.0
	60	82.7	81.7	101.2
B	0	13.7	–	–
	20	33.4	33.7	99.1
	40	54.5	53.7	101.5
	60	74.8	73.7	101.5
C	0	15.4	–	–
	20	35.7	35.4	100.8
	40	56.7	55.4	102.3
	60	76.1	75.4	100.9

Egg SM standard solution was added to the lipid extract. The concentration of SM was measured by the enzymatic fluorometric assay.

E1 (15 μL) containing 320 U/mL phospholipase D, 2.4 mM $CaCl_2$, 50 mM NaCl and 50 mM Tris−HCl (pH 7.4) was added to the sample (15 μL) and incubated at 37 °C for 2 h. The reaction mixture was incubated at 96 °C for 3 min and centrifuged at 7,200 *g* for 5 min to remove the denatured enzyme. The supernatant (20 μL) was mixed with Regent E2 (80 μL) containing 12.5 U/mL tyramine oxidase, 3.125 U/mL peroxidase, 187.5 μM Amplex Red, 0.125 % Triton X-100, 50 mM NaCl and 50 mM Tris−HCl (pH 7.4). After a 30-min incubation at 37 °C, Amplex Red Stop Reagent (20 μL) was added. Fluorescence intensity was measured at 544/590 nm (excitation/emission) by a SpectraMax iD3 multi-mode microplate reader (Molecular Devices, San Jose, CA, USA). POPE dissolved in 1% Triton X-100 solution was used as the standard for measuring PE.

3. Results and discussion

3.1. Particle sizes of isolated lipoproteins

We isolated human plasma lipoproteins, VLDL, LDL and HDL, by differential ultracentrifugation as described previously (Carlson, 1973; Chunta et al., 2016; Chunta et al., 2018). Non-denaturing polyacrylamide gradient gel electrophoresis and gel filtration chromatography have been widely used to characterize the isolated lipoprotein fractions (Gambert et al., 1990; Innis-Whitehouse et al., 1998). In addition, the dynamic light scattering technique has been established to determine the lipoprotein particle sizes, and the lipoprotein sizes determined by dynamic light scattering have been strongly correlated with those determined by non-denaturing polyacrylamide gradient gel electrophoresis and have been consistent with the gel filtration elution profiles (Alexandre et al., 2015; Chandra et al., 2016; Lima and Maranhao, 2004; O'Neal et al., 1998; Sakurai et al., 2010). In this study, to characterize the isolated lipoprotein fractions, we determined the size distribution of lipoprotein particles by dynamic light scattering measurement (Fig. 1a-c). The volume-weighted mean diameters of VLDL, LDL and HDL were 39.1, 19.6 and 11.7 nm, respectively, suggesting that the lipoprotein fractions were sufficiently isolated. Because it is difficult to strictly separate the particles at certain diameters by centrifugation procedures, the size distributions showed partial overlaps between lipoprotein fractions.

3.2. Assay reproducibility

We have previously established enzymatic fluorometric assays for quantifying PC, PE and SM (Morita et al., 2012b; Morita et al., 2010). To evaluate the reproducibility of the assays for PC, PE and SM in lipoproteins, the within-run and between-run precision was assessed. VLDL, LDL and HDL samples from three different human plasma samples were prepared to assess the within-run and between-run coefficients of variation (CV, n = 10). To determine the within-run CVs, each sample was measured in 10 replicates per run. By the enzymatic fluorometric assay, the PC contents in VLDL, LDL and HDL were 112–250, 547–1002 and 412−504 nmol/mL plasma, respectively (Table 1). The within-run CVs of the PC measurements were 1.6–2.1 % for VLDL, 1.5–2.6 % for LDL and 1.6–2.8 % for HDL. The PE contents in VLDL, LDL and HDL were 5–16, 26–47 and 16−42 nmol/mL plasma, respectively. The within-run CVs of the PE measurements for VLDL, LDL and HDL were 1.1–2.4 %, 1.3–1.6 % and 1.6–2.0 %, respectively. The SM contents in VLDL, LDL and HDL were 14–21, 166–294 and 70−85 nmol/mL plasma, respectively. The within-run CVs of the SM assays for VLDL, LDL and HDL were 0.9–2.2 %, 1.5–2.3 % and 1.2–1.4 %, respectively. To determine the between-run CVs, each sample was assayed in duplicate once daily for 10 days using the enzymatic fluorometric quantification with the same reagent lots. As depicted in Table 2, the between-run CVs of the PC measurements were 2.8–4.0 % for VLDL, 2.0–4.7 % for LDL and 2.7–3.4 % for HDL. By the PE enzymatic assay, the between-run CVs for VLDL, LDL and HDL were 2.2–4.4 %, 2.1–4.0 % and 2.8–4.5 %, respectively. The between-run CVs of the SM assays for VLDL, LDL and HDL were 2.2–3.2 %, 2.6–3.0 % and 1.6–3.3 %, respectively. The between-run CVs of the enzymatic fluorometric assays were relatively high compared with the corresponding within-run CVs. The contents of PC, PE and SM in lipoproteins tended to slightly decrease from day 1 to day 10, although, to avoid repeated freeze-thaw cycles, the sample solution was separated into ten sample tubes and frozen at −20 °C immediately after preparation. Therefore, we recommend the measurements be performed on the day of sample preparation.

3.3. Assay linearity

We have previously reported that, for the enzymatic fluorometric assays, the detection limits are 1 μM PC, 1 μM PE and 0.5 μM SM, and the upper limits are 150 μM PC, 250 μM PE and 100 μM SM (Morita et al., 2012b; Morita et al., 2010). Next, we investigated the linearity of the assays for PC, PE and SM in VLDL, LDL and HDL. The VLDL, LDL, and HDL lipid extracts from different human plasma samples were sequentially diluted in 1% Triton X-100 solution within the measurable ranges (1–150 μM PC, 1–250 μM PE and 1–100 μM SM). As shown in Fig. 2a-i, well-fitted regression lines were produced. The PC assays for VLDL, LDL and HDL demonstrated good linearity to 117.3 μM ($r > 0.999$), 123.6 μM ($r > 0.998$) and 126.4 μM ($r > 0.999$), respectively (Fig. 2a-c). The PE assays also provided good linearity ranges of 0–108.1 μM in VLDL ($r > 0.998$), 0–158.0 μM in LDL ($r > 0.998$) and 0–148.7 μM in HDL ($r > 0.999$) (Fig. 2d-f). In the SM assays, excellent linearity ranges of 0–68.6

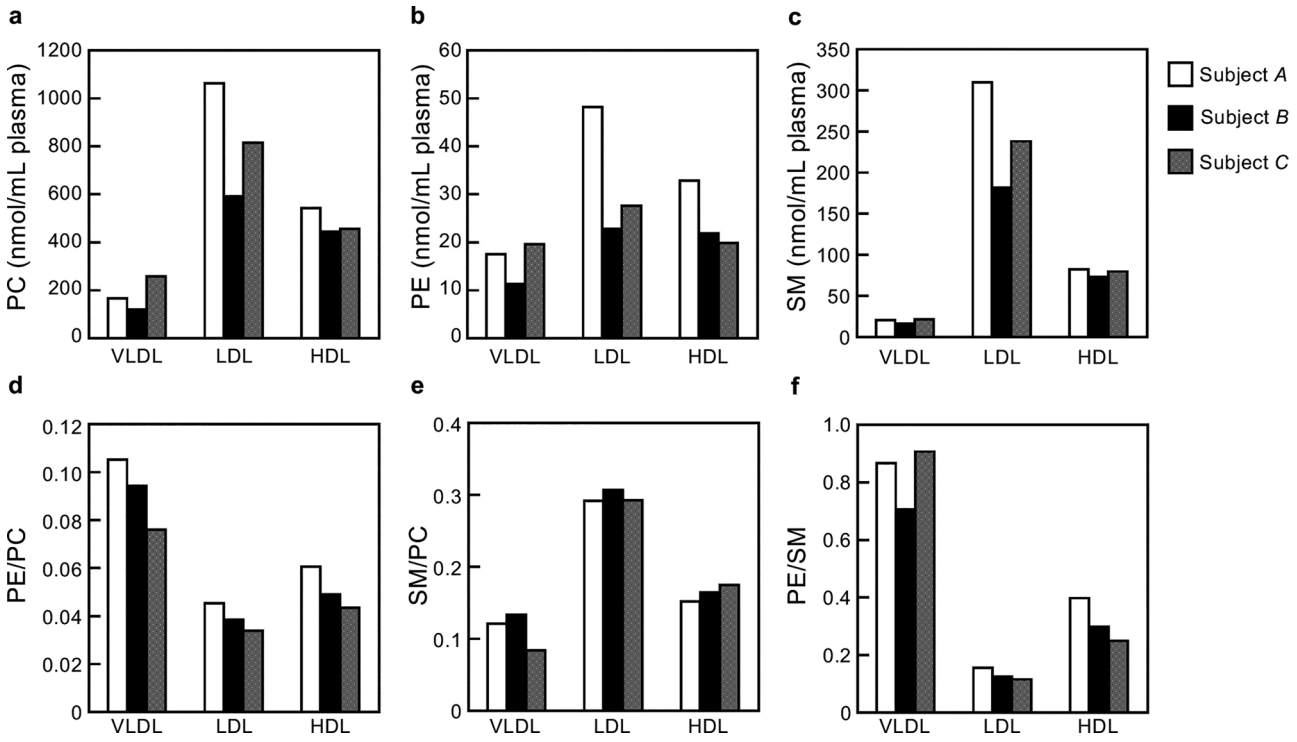

Fig. 3. Phospholipid composition in human plasma VLDL, LDL and HDL. VLDL, LDL and HDL were isolated from plasma collected from three different subjects (subject *A*, white bars; subject *B*, black bars; subject *C*, gray bars) by the ultracentrifugation method. The concentrations of PC (**a**), PE (**b**) and SM (**c**) in VLDL, LDL and HDL were measured by the enzymatic fluorometric assays, and the ratios of PE/PC (**d**), SM/PC (**e**) and PE/SM (**f**) were calculated.

μM in VLDL ($r > 0.999$), 0–94.6 μM in LDL ($r > 0.999$) and 0–75.5 μM in HDL ($r > 0.999$) were obtained Fig. 2g-h). Hence, an appropriate sample dilution is required for the phospholipid measurements when the sample contains higher concentrations of phospholipids than the upper limits.

3.4. Assay recovery

We assessed the accuracy of these assays by recovery studies. The method of Folch is one of the most widely used procedures to extract lipids (Folch et al., 1957). We have previously demonstrated that PC, PE and SM are almost completely recovered by the Folch extraction method (Tsuji et al., 2021). After the Folch method, the recovery rates of PC, PE and SM are 97.6 %, 99.5 % and 97.4 %, respectively (Tsuji et al., 2021). In this study, to confirm the recovery of PC, PE and SM assays, the known concentrations of standard solutions (0, 40, 80 and 120 μM) were added to the lipoprotein lipid extracts (1:1 ratio by volume). The VLDL, LDL and HDL samples from three different plasma samples were tested. The recovery of PC added to the VLDL, LDL and HDL lipid extracts at concentrations of 20−60 μM was 97.4–101.8 %, 95.2–102.4 % and 95.6–101.3 %, respectively (Table 3). The recovery of PE added to the VLDL, LDL, and HDL lipid extracts was 97.9–103.0 %, 96.9-102.3 % and 97.9–102.1 %, respectively (Table 4), and that of SM added to the VLDL, LDL, and HDL lipid extracts was 97.2–102.2 %, 97.4–102.3 % and 97.1–102.3 % (Table 5). Therefore, in the PC, PE and SM assays, there was no interference of hydrophobic compounds extracted from human plasma lipoproteins.

3.5. Phospholipid composition in human plasma lipoproteins

By using the enzymatic fluorometric assays, we determined the concentrations of PC, PE and SM in VLDL, LDL and HDL isolated from the plasma samples collected from three different subjects (Fig. 3). The lipoprotein phospholipid concentration was normalized with the volume of plasma (nmol/mL plasma), but not with the protein concentration, because the protein composition and concentration in each lipoprotein fraction may differ between individuals. In all samples, the concentrations of PC, PE and SM were the highest in LDL (823.4 ± 135.5, 32.9 ± 7.8 and 243.4 ± 37.0 nmol/mL plasma, mean ± S.E., n = 3, respectively), followed in descending order by HDL (481.5 ± 30.8, 24.9 ± 4.0 and 78.5 ± 2.7 nmol/mL plasma, respectively) and VLDL (181.6 ± 40.2, 16.2 ± 2.5 and 19.3 ± 1.6 nmol/mL plasma, respectively) (Fig. 3a-c). However, we caution that the lipoprotein phospholipid concentration per plasma volume may be affected by the isolation rate of the lipoprotein subclass from plasma, which is difficult to determine. In addition to the lipoprotein phospholipid concentration (nmol/mL plasma), we determined the relative amount of each phospholipid, PE/PC, SM/PC or PE/SM, in each lipoprotein fraction, which appears to be largely independent of the lipoprotein isolation rate. As shown in Fig. 3d and e, the SM/PC ratio in LDL (0.297 ± 0.005, mean ± S.E., n = 3) was higher than that in VLDL (0.113 ± 0.015), whereas the PE/PC ratio was lower in LDL (0.039 ± 0.003) than in VLDL (0.092 ± 0.009). In HDL, the ratios of PE/PC and SM/PC were 0.051 ± 0.005 and 0.164 ± 0.006, respectively (Fig. 3d and e). The PE/SM ratio was in the order VLDL (0.827 ± 0.062) > HDL (0.315 ± 0.044) > LDL (0.132 ± 0.012) (Fig. 3f).

Jiang et al. have reported that the plasma SM levels and SM/(PC + SM) ratios are higher in patients with coronary artery disease than in control subjects (Jiang et al., 2000). Thus, plasma SM levels and SM/(PC + SM) ratios have been suggested to be an independent risk factor of coronary artery disease (Jiang et al., 2000). In the arterial wall, SMase hydrolyzes SM to ceramide in LDL and enhances the uptake of LDL by macrophages, which leads to the formation of foam cells (Morita, 2016). SM on the particle surface reduces lipolysis by lipoprotein lipase and the hepatocyte uptake mediated by apolipoprotein E, which may delay the clearance of VLDL from circulation (Arimoto et al., 1998; Morita et al., 2003; Saito et al., 2000). Enrichment of HDL with SM suppresses the cholesterol esterification mediated by lecithin:cholesterol acyltransferase (Subbaiah and Liu, 1993). We therefore consider it important to quantify PC, PE and SM in each lipoprotein fraction.

In mouse plasma VLDL, the amount of PC is approximately 30-fold larger than that of PE (PE/PC ≈ 0.03) (Zhao et al., 2009). After the

injection of the lipase inhibitor Poloxamer 407, the PE/PC ratio in VLDL increases to approximately 0.08, suggesting that the lipases preferentially hydrolyze PE relative to PC (Zhao et al., 2009). The SM/PC ratio increases during the conversion from VLDL to LDL, which may be due to the resistance of SM against lipoprotein lipase and hepatic lipase and possibly to the slow movement of SM between lipoprotein particles (Myher et al., 1989; Subbaiah and Liu, 1993). In humans, the PE/PC ratios have been reported to be 0.06 in VLDL, 0.03−0.04 in LDL, 0.03−0.06 in HDL (Dashti et al., 2011; Subbaiah et al., 1989; Subbaiah and Liu, 1993). The previously reported ratios of SM/PC in human lipoproteins are 0.09−0.23 in VLDL, 0.21−0.48 in LDL and 0.08−0.38 in HDL (Dashti et al., 2011; Schissel et al., 1998; Subbaiah et al., 1989; Subbaiah and Liu, 1993; Windler et al., 1986; Yang and Subbaiah, 2015). These values have been obtained from small sample size studies (n = 1–14) by the conventional methods using thin-layer chromatography, which are less sensitive than the enzymatic fluorometric assays. The SM/PC ratio in LDL (~0.3) obtained in this study is relatively low compared with the ratios in previous reports, which may be partly caused by differences in the quantification methods. To establish the standard values and to detect abnormalities in the lipoprotein metabolism, we need to measure PC, PE and SM in many lipoprotein samples using the enzymatic fluorometric assays.

4. Conclusion

In conclusion, we optimized and validated the enzymatic fluorometric assays for the quantification of PC, PE and SM in VLDL, LDL and HDL. These enzymatic fluorometric assays enable more precise and accurate quantifications of phospholipid classes in lipoproteins. Although the physiological and pathological roles of PC, PE and SM in lipoproteins remain largely unknown, these enzymatic fluorometric assays will be helpful in clarifying the functions of PC, PE and SM in lipoprotein metabolism and in exploring their relationships with diseases including atherosclerosis.

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Author contributions

T. Tsuji designed the research, performed the experiments, analyzed the data and wrote the original manuscript. S.M. designed the research, analyzed the data, reviewed and edited the manuscript. T.Y. and T. Terada provided scientific expertise. All authors discussed the results and reviewed the manuscript, and approved its submission for publication.

Transparency document

The Transparency document associated with this article can be found in the online version.

Declaration of Competing Interest

The authors declare no competing interests.

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