Rapid, specific, and sensitive measurements of plasma sphingomyelin and phosphatidylcholine

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Abstract **Sphingomyelin (SM) and phosphatidylcholine (PC) are two major phospholipids on plasma lipoproteins. Their concentration is classically measured by lipid extraction, thin-layer chromatography, and phosphate determination on separated SM or PC spots. Here, we describe two rapid, specific, and sensitive enzymatic measurements for both phospholipids. Plasma was incubated with bacterial sphingomyelinase (for SM measurement) or bacterial PC-specific phospholipase D (for PC measurement), alkaline phosphatase, choline oxidase, peroxidase, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline, and 4-aminoantipyrine for 45 min. A blue dye, with an optimal absorption at 595 nm, was generated. PC levels did not influence SM measurement and vice versa. The linear range for the SM measurement was 0.5–5 μg, and that for PC was 2.5–20 μg. The mean percentage recovery was 98.0 ± 5.2% for SM and 96.6 ± 3.8% for PC. The interassay coefficient of variation of the assay was 1.7 ± 0.05% for SM and 3.1 ± 0.13% for PC. These two new methods are amenable to automation and can be adapted for large-scale, high-throughput assays.—**Hojjati, M. R., and X-C. Jiang. **Rapid, specific, and sensitive measurements of plasma sphingomyelin and phosphatidylcholine. *J. Lipid Res.* 2006.** 47: **673–676.**

Supplementary key words phospholipid measurement • large-scale and high-throughput assays • enzymatic measurements

Besides cholesterol and triglycerides, plasma also contains phospholipids. Phosphatidylcholine (PC) and sphingomyelin (SM) are two major phospholipids, the former comprising ~70% and the latter ~20% of the total phospholipids in the circulation (1). In a human case-control study, it was found that both plasma SM and SM/PC ratio are independent risk factors for coronary heart disease (2). It has been known for some time that SM accumulates in atheromas in human and animal models (3, 4). LDL extracted from human atherosclerotic lesions is much richer in SM than LDL from plasma (5, 6). Plasma SM levels in apolipoprotein E knockout mice are four times higher than those in wild-type mice (7), and this may partly explain the increased atherosclerosis in these animals (8). The SM/PC ratio was increased five times in VLDL from hypercholesterolemic rabbits (9). Recently, we and others demonstrated for the first time that administration of myriocin (an inhibitor of SM synthesis) into apolipoprotein E knockout mice dramatically decreased SM, increased PC, thus decreasing the SM/PC ratio in the plasma, and significantly decreased the atherosclerotic lesion area (10, 11). These data suggest that SM might play a promoting role, whereas PC might play a preventive role, in the development of atherosclerosis, and their measurement might provide new insights into atherogenesis in humans and in various mouse models as well. Although the importance of both phospholipids is very obvious, there are no simple, rapid, sensitive, and high-throughput methods for their measurement. Classically, plasma SM and PC were measured by lipid extraction, thin-layer chromatography, and phosphate determination on separated SM or PC spots. This method is time-consuming and not sensitive. Here, we describe two rapid, specific, and sensitive enzymatic measurements for both phospholipids.

MATERIALS AND METHODS

Reagents

Sphingomyelinase (SMase; S-8889), alkaline phosphatase (P5521), choline oxidase (C5896), peroxidase (P1432), and 4-aminoantipyrine (A4382) as well as standard SM (S-0756) and standard PC (P-3556) were purchased from Sigma-Aldrich. PC-specific phospholipase D was purchased from Biomol International. DAOS [*N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline, sodium salt] was purchased from Dojindo Molecular Technologies, Inc.

Abbreviations: DAOS, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline; PC, phosphatidylcholine; SM, sphingomyelin; SMase, sphingomyelinase.

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Manuscript received 11 November 2005 and in revised form 5 December 2005.

Published, JLR Papers in Press, December 21, 2005.
DOI 10.1194/jlr.D500040-JLR200

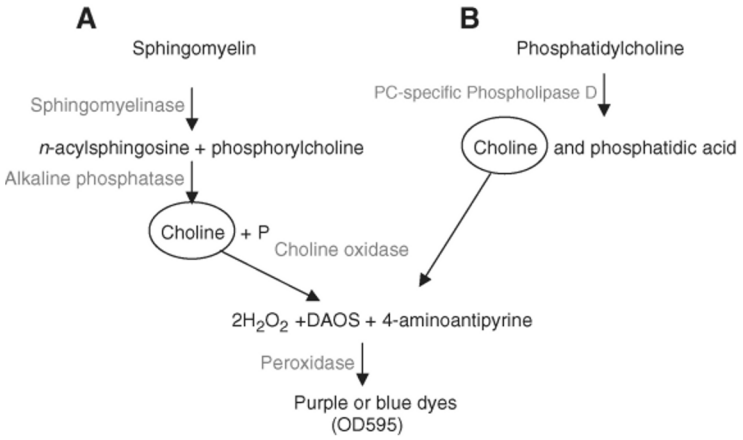

Fig. 1. Strategy for sphingomyelin (SM) and phosphatidylcholine (PC) measurements. A: Sphingomyelinase (SMase) catalyzes the hydrolysis of SM to phosphorylcholine. Alkaline phosphatase catalyzes in the second step, in which phosphorylcholine produces choline. Oxidation of choline is the next step, catalyzed by choline oxidase. This reaction produces two hydrogen peroxides. The last step catalyzes by peroxidase, which produces a blue dye that can be measured. B: PC-specific phospholipase D catalyzes the hydrolysis of PC to choline and phosphatidic acid. The rest of the reactions are similar to the SM measurement. OD, optical density.

SM measurement

There were four steps for the enzymatic measurement of plasma SM levels (**Fig. 1A**): *1*) bacterial SMase hydrolyzed SM to phosphorylcholine and *n*-acylsphingosine; *2*) alkaline phosphatase generated choline from phosphorylcholine; *3*) choline was used to generate hydrogen peroxide in a reaction catalyzed by choline oxidase; and *4*) hydrogen peroxide was used together with DAOS, 4-aminoantipyrine, and peroxidase, as a catalyst, to generate a blue dye with an optimal absorption at 595 nm. The reaction buffer was 0.05 M Tris-HCl with 0.66 mM calcium chloride, pH 8. Enzyme concentrations in a 50 ml reaction buffer were as follows: 25 units of SMase, 500 units of alkaline phosphatase, 25 units of choline oxidase, and 1,000 units of peroxidase. DAOS concentration was 0.73 mM, and 4-aminoantipyrine concentration was 0.73 mM. Five microliters of plasma was added to a 100 μl reaction buffer containing enzymes, and after 45 min of incubation at 37°C, the absorption was measured at 595 nm on a spectrophotometric plate reader. The developed color remained constant after the incubation time. Standard SM solution (50 mg/dl) preparation (5 mg of SM) was dissolved in 10 ml of 2% Triton X-100 ethanol solution.

PC measurement

There were three steps for the enzymatic measurement of plasma PC levels (Fig. 1B): *1*) bacterial phospholipase D (specific for PC, no reaction with SM) hydrolyzed PC to choline and phosphatidic acid; *2*) choline was used to generate hydrogen peroxide in a reaction catalyzed by choline oxidase; and *3*) hydrogen peroxide was used together with DAOS, 4-aminoantipyrine, and peroxidase, as a catalyst, to generate a blue dye with an optimal absorption at 595 nm. The reaction buffer was 0.05 M Tris-HCl with 0.66 mM calcium chloride, pH 7. Enzyme concentrations in a 50 ml reaction buffer were as follows: 6,000 units of phospholipase D (added at the time of measurement), 25 units of choline oxidase, and 1,000 units of peroxidase. DAOS concentration was 0.73 mM, and 4-aminoantipyrine concentration was 0.73 mM. Five microliters of plasma was added to a 100 μl reaction buffer containing enzymes, and after 45 min of incubation at 37°C, the absorption was measured at 595 nm. The developed color remained constant after the incubation time. Standard SM solution (100 mg/dl) preparation (10 mg of PC) was dissolved in 10 ml of 2% Triton X-100 ethanol solution.

Total phospholipid measurement

The total choline-containing phospholipids (PC and SM) in plasma were measured with a commercial kit (Wako Pure Chemical).

RESULTS

Enzymatic measurement of plasma SM or PC levels was carried out using the novel four-step or three-step procedure (Fig. 1). As indicated in **Fig. 2**, the linear range for the SM measurement was 0.5–5 μg, and that for PC was 2.5–20 μg (Fig. 2). We also measured the SM and PC concentrations in different amounts of pooled human plasma and found that the linear range for both assays was 2.5–10 μl (**Fig. 3**). However, a much better linear fit was obtained without forcing the curve through zero, suggesting that these concentrations may be slightly overestimated. The linear range for mouse plasma SM or PC assay was similar to that for humans (data not shown).

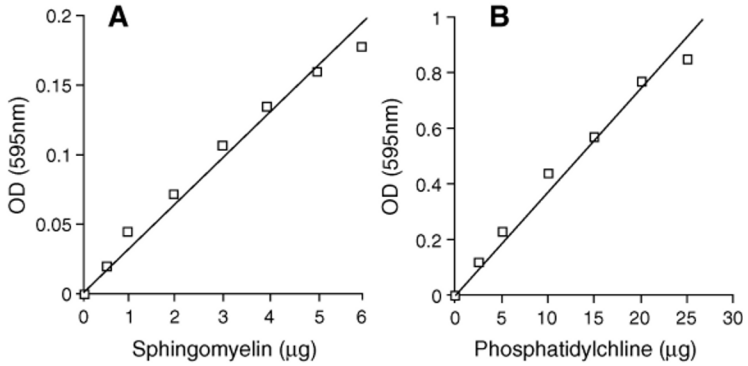

Fig. 2. Standard curves for the SM and PC measurements. Different amounts of SM or PC standard solution supplemented with saline to 20 μl were incubated with a 100 μl reaction buffer at 37°C for 45 min, and absorption was measured at 595 nm. A: Standard curve for SM. B: Standard curve for PC. OD, optical density.

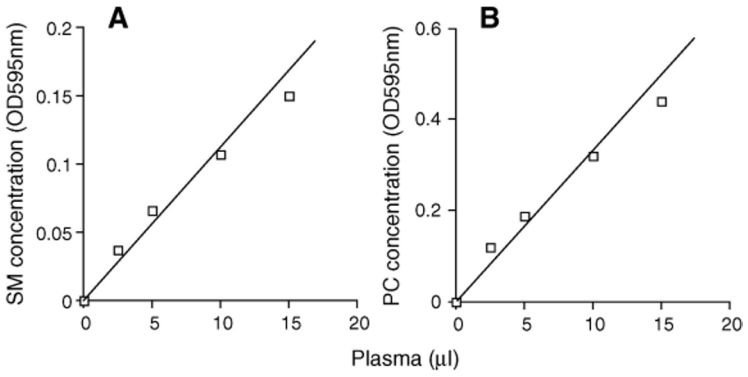

Fig. 3. Linear ranges of plasma SM and PC measurements. Pooled human plasma was used. Different amounts of the plasma supplemented with saline to 20 μl were incubated with a 100 μl reaction buffer at 37°C for 45 min, and absorption was measured at 595 nm. A: Plasma linear range for SM measurement. B: Plasma linear range for PC measurement. OD, optical density.

We next sought to determine the accuracy of both methods by recovery studies in which known quantities of SM or PC were added to a pooled human plasma sample. The results are presented in **Table 1**. The mean percentage recovery of SM was 98.0% and that of PC was 96.6%.

Because both methods are very similar except for the first step (Fig. 1), it is likely that both measurements would interfere with each other. To investigate the specificity for the SM method, we used standard PC as a substrate and vice versa, and we found that there was no crossing measurement in either method (data not shown).

Hemolysis always occurs during blood collection. Because the plasma SM concentration is significantly lower than the cholesterol and PC concentrations, the hemolytic plasma may significantly interfere with the absorption reading of the assay. To investigate this effect, we used 10 μl of low (optical density = 0.1–0.2), medium (optical density = 0.2–0.3), and high (optical density = 0.3–0.4) hemolytic plasma samples, monitored by absorption at 415 nm (12), incubated them with the SM assay solution but without SMase at 37°C for 45 min, and then measured their absorption at 595 nm. We found that hemolytic samples showed the same background reading as the blank, indicating that hemolysis did not interfere with the SM assay (**Fig. 4**).

The precision of both assays was established by analyzing SM and PC levels in a pooled human plasma sample 20 times. The interassay coefficient of variation of the SM assay was 1.7 ± 0.05%, and that of the PC assay was 3.1 ± 0.13%.

To validate our novel SM and PC assays, we measured total choline-containing phospholipid (SM and PC) levels in mouse plasma using a commercial kit (Wako Pure Chemical) and then compared the results with those obtained by adding SM and PC concentrations measured by our new methods. We found that the two approaches correlated well (r = 0.91, n = 17) (**Fig. 5**). The mouse (C57BL/6) plasma SM concentration was 33 ± 10 mg/dl, the PC concentration was 134 ± 21 mg/dl, and the PC/SM ratio was 4.1 ± 1.0 (**Table 2**). These results were comparable with those obtained by the classical method (7), by which the SM concentration was 24 ± 5 mg/dl and the PC concentration was 125 ± 12 mg/dl in the same inbred mice.

DISCUSSION

Classically, SM and PC were measured by four steps (13, 14): *1*) lipid extraction; *2*) thin-layer chromatography; *3*) SM and PC extraction from corresponding spots on the thin-layer chromatography plate; and *4*) quantification of phosphate in each extraction. The whole procedure is time-consuming (requiring 2 days of work) and not sensitive (requiring at least 50 μl of plasma). Although there is a simple method for measuring total choline-containing phospholipids (Wako Pure Chemical), there is no corresponding method for direct SM and PC measurements. In this study, we developed two rapid, specific, and sensitive assays for plasma SM and PC measurements.

TABLE 1. Plasma SM and PC recovery studies

	SM Concentration			PC Concentration		
Tube No.	Observed[a]	Added	Recovery	Observed[a]	Added	Recovery
	mg/dl		%	*mg/dl*		%
1	16	15	107	31	30	103
2	29	30	97	57	60	95
3	43	45	96	86	90	96
4	58	60	97	115	120	96
5	70	75	3	139	150	93

PC, phosphatidylcholine; SM, sphingomyelin. To 500 μl of pooled human plasma sample, 3, 6, 9, 15, and 18 μl of standard SM solution (2.5 g/dl in 2% Triton X-100 ethanol solution) or 3, 6, 9, 15, and 18 μl of standard PC (5 g/dl in 2% Triton X-100 ethanol solution) were added in tubes 1–5, respectively. Five microliters of these plasma samples was used for SM and PC measurements. The endogenous SM concentration of the pooled plasma was 45 mg/dl, and the endogenous PC concentration was 139 mg/dl.

[a]Observed concentration = increased concentration in the pooled plasma.

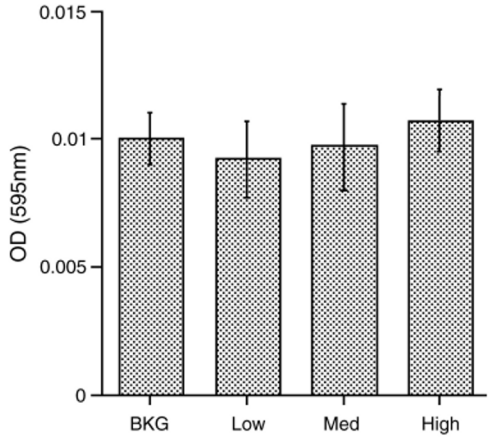

Fig. 4. The effect of hemolysis on optical density (OD) readings at 595 nm. Ten microliters of low, medium, and high hemolytic mouse plasma samples, monitored by absorption at 415 nm, was incubated with 100 μl of the SM assay solution but without SMase at 37°C for 45 min, and absorption was measured at 595 nm. BKG, background; Low, low hemolysis; Med, medium hemolysis; High, high hemolysis. Values are means ± SD (n = 3).

Our assay system is close to the commercial kit (Wako Pure Chemical), except for the first step and the last step. In the first step, a nonspecific phospholipase D is used to hydrolyze all choline-containing phospholipids (PC and SM) in the kit, whereas SMase or PC-specific phospholipase D is used in our assays; as a result, the kit cannot, but our methods can, distinguish SM and PC. In the last step, phenol is used to develop a red quinine pigment (with an optimal absorption at 505 nm) in the kit, whereas DAOS is used in our assays to develop a blue dye (with an optimal absorption 595 nm). Because hemolytic plasma could significantly influence the absorption at 505 nm (data not shown), we used DAOS as a color developer in our assays.

From our experience, only PC-specific phospholipase D purchased from Biomol International can be used in our PC assay, because most commercially available phospholipase D is contaminated with SMase activity. Most of the SMase available from Sigma-Aldrich can be used in our assay. All alkaline phosphatase, choline oxidase, and peroxidase available from Sigma-Aldrich are usable in both assays.

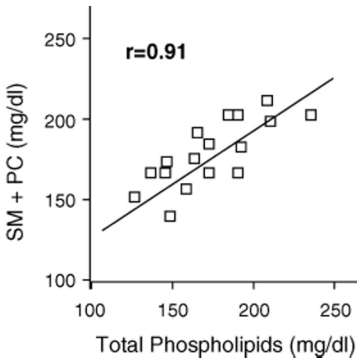

Fig. 5. Comparison of the new methods for mouse plasma SM and PC measurement with a commercial kit (Wako Pure Chemical) that measures total choline-containing phospholipids. $r = 0.91$, n = 17.

TABLE 2. Comparison of the new methods for mouse (C57BL/6) plasma SM and PC measurements with a commercial kit that measures total choline-containing phospholipids

SM[a]	PC[a]	PC + SM	PC/SM	PL[b]
mg/dl				*mg/dl*
33 ± 10	134 ± 31	165 ± 29	4.1 ± 1.0	177 ± 37

Values are means ± SD (n = 17).
[a] Measured by the new methods.
[b] Measured by a commercial kit (Wako Pure Chemical).

In conclusion, our novel methods for plasma SM and PC measurement are simple, rapid, specific, sensitive, and high-throughput. They are suitable for larger scale clinical sample measurements or drug screening and may be adapted for tissue SM and PC measurements.

This work was supported by National Institutes of Health Grants HL-64735 and HL-69817.

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