Specific, rapid, and sensitive enzymatic measurement of sphingomyelin, phosphatidylcholine and lysophosphatidylcholine in serum and lipid extracts

[Full paper]

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Abbreviations: PL, phospholipids; DAOS, N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline; PC, phosphatidylcholine; SM, sphingomyelin; SMase, sphingomyelinase; VLDL, very low density lipoprotein; LDL, low-density lipoprotein; HDL, high density lipoprotein; PLD, phospholipase D; LCAT, lecithin cholesterol acyltransferase; 2,5-DHB, 2,5-dihydroxybenzoic acid; TLC, Thin-layer liquid chromatography; HPLC, high performance liquid chromatography.

ABSTRACT

Objective: Human serum sphingomyelin (SM) and phosphatidylcholine (PC) play important roles in the development of atherosclerosis. However, there are no rapid and sensitive methods for SM and PC measurement. The present report describes a novel enzymatic method for measuring SM, PC and lysophosphatidylcholine (lyso-PC) levels in plasma and lipid extracts.

Design and Methods: The total choline-containing phospholipids (total PL), SM and PC were measured using a two-reagent system involving specific enzymes for choline-based phospholipids. The procedure was performed using either microplate or automatic analyzer technology. The concentration of lyso-PC was calculated by subtracting the concentration of SM plus PC from the total PL concentration.

Results: Assay results showed linear correlations between sample concentration and absorbance. The within-run and between-run coefficients of variation for PC, SM, and lyso-PC concentrations were 2.0-4.4% for the microplate analyzer and 0.9-2.9% for the automatic analyzer. Analysis of normal human serum showed that the total PL concentration strongly correlated with the SM plus PC concentration (r=0.9850). There were moderate correlations between serum PC and SM levels (r=0.6228) and between

serum PC and lyso-PC levels (r=0.7806). SM, PC, and lyso-PC levels in normal human serum (n=50) were 0.54 ± 0.07, 1.99 ± 0.22 and 0.60 ± 0.15 mmol/l, respectively.

Conclusion: The present enzymatic method allowed for rapid, simple, and accurate measurement of SM, PC, and lyso-PC levels in lipid extracts and in serum. The method is suitable for both microplate and automatic analyzer assays.

INTRODUCTION

The choline-containing phospholipids sphingomyelin (SM), phosphatidylcholine (PC), and lysophosphatidylcholine (lyso-PC) are the three major phospholipids found in humans [1]. These molecules comprise the major outer surface phospholipids of cell membranes and are also the major phospholipids of plasma lipoproteins [2]. Furthermore, SM, PC, and lyso-PC play important roles in the modulation of membrane-enzyme activity, cholesterol homeostasis, and in signaling pathways [3].

SM has been shown to accumulate in atheromata, and the major source of such SM is plasma lipoproteins [4]. Human plasma SM levels are positively and independently associated with cardiovascular disease, suggesting plasma SM levels may be a marker

of atherogenic remnant lipoprotein accumulation and may be a predictor of outcomes in acute coronary syndrome patients [5, 6]. Plasma SM levels are elevated in human familial hyperlipidemia [7] and in animal models of atherosclerosis [8]. Enrichment of the diet with sphingolipids has proatherogenic effects [9].

Lyso-PC is produced by the action of phospholipase A_2 (PLA_2) or lecithin cholesterol acyltransferase (LCAT) on PC. Lyso-PC regulates a variety of biological processes including cell proliferation, tumor cell invasiveness, and inflammation [10], and plays an etiological role in atherosclerosis as a component of oxidized low-density lipoprotein (oxLDL) [11]. The SM : PC ratio in lipoproteins appears to be an important factor in their susceptibility to sphingomyelinase (SMase) [12]. Low-density lipoprotein (LDL) extracted from human atherosclerotic lesions is much richer in SM than that from plasma [13]. High plasma SM levels and a high SM : PC ratio may be risk factors for atherosclerosis.

Accurate measurement of SM, PC, and lyso-PC levels in plasma lipoproteins and cell membranes is critical for investigating the mechanisms underlying lipid metabolism and in the diagnosis of arteriosclerosis. Measuring phospholipids using thin layer chromatography (TLC) [14] or high-performance liquid chromatography (HPLC) [15]

is complicated. MALDI-TOF-MS can provide quantitative and rapid assessment of the ratio of phospholipids that differ only in fatty acid composition (i.e., PC or SM [16]) [17]. However, it is difficult to perform quantitative comparisons of molecules that differ in basic configuration (i.e., PC and SM) using MALDI-TOF-MS [18]. Previously reported methods for measuring SM and PC are time-consuming or not sufficiently precise [18, 19].

It is important to measure the level of phospholipids fractions in serum and peripheral blood cell to study the lipid metabolism and the mechanism in the development of atherosclerosis. The present report describes an enzymatic method of measuring SM and PC levels in human plasma and lipid extracts. Lyso-PC levels were calculated by subtracting the concentrations of SM and PC from the concentration of total choline-containing phospholipids (total PL). The procedure is rapid, simple, and accurate, and can be used in microplate or automatic analyzer assays.

Materials and Methods

Subjects

Serum was collected from volunteers (n=50) after obtaining informed consent and approval from the Ethical Review Board of the Shinshu University School of Medicine. All blood samples for the measurement of serum level were obtained after a 12- 14 hr fast and collected in tube.

Chemical reagents and materials

PC-specific phospholipase D (10000U/0.5 ml) was purchased from BIOMOL International, L.P. (Plymouth Meeting, PA). Sphingomyelinase (25 U/ml) was purchased from ICN Biomedicals (Irvine, CA), alkaline phosphatase (48600 U/ml) was purchased from Oriental Yeast (Osaka, Japan), and choline oxidase, horseradish peroxidase (100 U/mg), egg yolk 3-sn-phosphatidylcholine as PC-standard, 4-aminoantipyrine (MW: 203.24), Triton X-100, methanol, chloroform, hydrochloric acid, and aqueous ammonia were purchased from Wako Chemical Co. (Osaka, Japan). DAOS [N-ethyl-N- (2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline, sodium salt] (MW: 341.36) was purchased from Dojindo Laboratories (Tokyo, Japan), bovine albumin (F-V) was purchased from Nakalai Tesque (Kyoto, Japan), 2,5-Dihydroxybenzoic acid (2,5-DHB) was purchased from Sigma Chemical Co. (St. Louis, MO), and egg

sphingomyelin as SM-standard was purchased from NOF Corporation (Tokyo, Japan).

Specificity of measurement

The stages of the SM and PC enzymatic assays are illustrated in Fig. 1. SM was hydrolyzed to *n*-acylsphingosine and phosphocholine using sphingomyelinase, and choline was generated from phosphocholine using alkaline phosphatase (ALP) (Fig. 1-A). PC was hydrolyzed to choline using PC-specific phospholipase D (PLD, Fig. 1-B). PC-specific PLD hydrolyzes only choline PCs, and does not react with SM or lyso-PC. For measurement of total phospholipids, total choline from phospholipids was generated by hydrolysis using non-specific PLD (a commercial kit from Wako Pure Chemical; Osaka, Japan). Choline generated from SM, PC, and lyso-PC was reacted with cholineoxidase to generate hydrogen peroxide. The hydrogen peroxide reacting with DAOS, 4-aminoantipyrine, and peroxidase in an oxidation condensation reaction generated a blue dye with optimal absorption at 595 nm.

Preparation of SM reagents

The SM assay procedure is illustrated in Figure 1. Measurements were performed using a two-reagent system. Reagent 1 (R-1) consisted of SMase (0.05 U), ALP (4.86 U), DAOS (2 mM), and 0.05% Triton X-100 in a buffer of 0.05 M Tris-HCl and 0.66 mM calcium chloride (pH 8). Reagent 2 (R-2) consisted of choline oxidase (0.07 U), peroxidase (3 U), and 4-aminoantipyrine (0.72 mM) in a buffer of 0.05 M Tris-HCl and 1 mM calcium chloride (pH 8). The SM standard solution contained sphingomyelin dissolved in 2% TritonX-100 in ethanol, and the concentration was adjusted to 14.2 mmol/l. This SM standard solution was diluted 10-fold in 2% Triton X-100 in ethanol and used at 1.42 mmol/l.

Preparation of PC reagents

The PC assay procedure is illustrated in Figure 1. Measurements were performed in a two-reagent system. Reagent 1 (R-1) consisted of PC-specific PLD (18 U), DAOS (2 mM), and 0.1% Triton X-100 in a buffer of 0.05 M Tris-HCl and 1 mM calcium chloride (pH 7.4). Reagent 2 (R-2) consisted of choline oxidase (0.07 U), peroxidase (3 U), and 4-aminoantipyrine (0.72 mM) in buffer of 0.05 M Tris-HCl and 1 mM calcium chloride (pH 7.4).

The PC standard solution contained PC (egg yolk) dissolved in 2% Triton X-100 in ethanol, and was adjusted to 26.4 mmol/l (stock solution). Stock solution was diluted 10-fold in 2% Triton X-100 in ethanol (microplate assay) or in H_20 (automatic assay), then adjusted to 1% BSA, and was used at a final concentration of 2.64 mmol/l.

Measurement protocol

The microplate method used flat-bottomed 96-well plastic plates. The automatic analyzer method used an Hitachi-7150 automatic analyzer for SM, PC, or total PL assay. For the microplate method, 5 µl (for PC assay) or 10 µl (for SM assay) sample or standard solution and 100 µl R-1 were added to 0.5 ml glass microtubes, and incubated for 10 min at 37°C. After incubation, 50 µl R-2 was added and incubated for 20 min at 37°C. Absorption was measured at 620 nm using a spectrophotometric plate reader.

For the automatic analyzer method, 5 µl sample or standard solution and 200 µl R-1 were added to the analyzer cells, and incubated for 5 min at 37°C. After incubation, 100 µl R-2 was added to the reaction cells and incubated for 5 min at 37°C. Absorption was measured at 600/700 nm (test/reference wavelength). Total PL (choline-containing phospholipids) in samples was measured using a commercial kit (Wako Pure Chemical)

in accordance with the manufacturer's protocol.

Preparation of human lipoproteins

Ultracentrifugation was accomplished using a Beckman TLA 100.3 rotor in an Optima TLX ultracentrifuge (Beckman Coulter, Fullerton, CA). Human high density lipoprotein (HDL: d. 1.063 – 1.21) were prepared using ultracentrifugation. After separation by ultracentrifugation, lipoprotein fractions were dialyzed in PBS overnight.

Mass spectrometry

Mass spectrometry measurements were performed as described previously using a MALDI-TOF MS (Voyager Elite XL; PerSeptive Biosystems, Framingham, MA) (18). The system utilized a pulsed nitrogen laser (emission, 337 nm; delay, 100 ns; acceleration voltage, 25 kV). The resolution of the ion peak was $M/\Delta M$ as determined using a resolution calculator from the GRAMS/386 software supplied with the instrument. To enhance the spectral resolution, the device was used in the reflector mode such that the total field-free time-of-flight distance was 3.0 m. In the positive ion mode with 2,5-DHB as the matrix, angiotensin I ($[M+H^+]$: 1296.69) and des-Arg-

bradykinin ([M+H^+]: 904.47) were used for instrument calibration. Five-point Savitsky-Golay smoothing was applied to the mass spectra. For MALDI-TOF MS analysis, 1.0 μl lipid solution and 1.0 μl matrix solution (10 mg 2,5-DHB in 1 ml chloroform/methanol, 2/1 v/v) were placed into a 0.4-ml glass microtube, and the mixture was then applied to a metal sample plate. After the sample had dried, the metal plate was inserted into the analyzer. The lipid mass was measured at an absorption laser intensity of approximately 2000.

RESULTS

1. Specificity of SMase and PLD

To evaluate the specificity of the hydrolases, human HDL were incubated with SMase or PC-specific PLD solutions at 37°C for 30 min, respectively. The phospholipids were then extracted using chloroform-methanol (C/M, 2:1 v/v) solution, the extract was dried under nitrogen gas, and dissolved again in C/M (2:1 v/v) solution. The extract was mixed with matrix agent (2,5-DHB), and the phospholipids analyzed using MALDI-TOF-MS. The data tracings show a dramatic reduction in the SM peaks at m/z 703.8 and 725.8 following incubation with SMase, and that an SM hydrolysate peak at m/z

520.6 was generated (Fig. 2-A, C). Similarly, there was a dramatic decrease in the PC peaks at m/z 758.7 to 828.7 following incubation with specific PLD, and the SM peaks was remained, and not detected SM hydrolysate peak (Fig. 2-B, D). These findings indicate that the assay conditions resulted in specific hydrolysis of SM or PC.

2. Microplate analyzer method

A calibration reaction was performed using PC or SM standard solutions to identify the region of a linear relationship between lipid concentration and absorbance (Fig. 3-A). Human plasma was then sequentially diluted with saline and samples were assayed for SM and PC. The regression line for SM passed linearly through all measurement points up to 0.9 mmol/l, while the PC regression line passed linearly through all points up to 2.2 mmol/l (Fig. 3-B).

3. Automatic analyzer method

The enzymatic reaction time course and concentration-response lines for serum SM and PC measured using the automatic analyzer method are shown in Figure 3. Absorbance values for SM and PC blank reaction samples were very low, while values

for serum were very high, almost reaching the reaction end-point by 10 min, 35 absorbance photometry point (Fig. 3-C) . Serial serum dilution with saline resulted in a linear relationship between lipid concentration and dilution for both SM and PC (Fig. 3-D). The linear relationships existed up to 0.9 mmol/l for SM, and 2.2 mmol/l for PC. A much better linear fit was obtained without forcing the curve through zero.

4. **Reproducibility of assay**

To determine the reproducibility of the assay, samples from human serum containing low, medium and high levels (sample number: I, II and III, respectively) of SM, PC and total choline-containing phospholipids (total PL) were repeatedly analyzed. The concentration of lyso-PC was calculated by subtracting the concentrations of SM and PC from the total PL concentration. For the microplate method, the within-run coefficients of variation (CV%, n=20) for low, medium and high concentrations of human serum were 2.3 - 3.6% for SM, 2.0 - 3.5% for PC, and 2.2 - 3.8% for lyso-PC. The overall coefficients of variation (n=10) were 2.4 - 4.1% for SM, 2.4 - 3.7% for PC, and 2.4 - 4.4% for lyso-PC (Table 1). The same sample preparations were used to test the reproducibility of the automatic analyzer method. The within-run coefficients of

variation (CV%, n=20) for low, medium and high concentrations of human serum were 1.0 - 1.6% for SM, 0.9% for PC, and 1.4 - 2.7% for lyso-PC. The overall coefficients of variation were 1.4 – 2.2% for SM, 1.0 - 1.1% for PC, and 1.8 - 2.9% for lyso-PC (Table 1). These results indicate that the optimized enzymatic assays for SM, PC, and lyso-PC using either microplate or automatic analyzer methods were highly reproducible.

5. Serum SM and PC levels in healthy subjects

The normal reference ranges for SM, PC, and lyso-PC in serum were obtained by assaying serum samples from 50 healthy human adults (age 21–63 years, 19 male and 30 female) using automatic analyzer method. The human serum SM, PC, and lyso-PC levels were 0.54 ± 0.07, 1.99 ± 0.22, and 0.60 ± 0.15 mmol/l respectively (mean ± SD), and the SM/PC and lyso-PC/PC ratios were 0.27 ± 0.03 and 0.30 ± 0.05, respectively (mean ± SD).

6. **Method comparison**

To evaluate the accuracy of the automatic analyzer method, the sum of the SM and PC levels was compared with the total PL value (i.e., SM, PC, and lyso-PC) for each

subject. Total PL, SM, and PC levels in fresh human sera from healthy subjects (n=50) were determined using the automatic analyzer method. The SM + PC values were graphed against the total PL values (Fig. 4). Regression and correlation analysis produced a graph where y = 0.7681x + 0.316, and r = 0.9850. These data indicate that sum of the SM and PC values correlated well with the total PL level.

7. Correlations between PC, SM and lyso-PC levels

The relationships between PC and SM levels, and between PC and lyso-PC levels, were examined. SM and PC serum levels for 50 healthy subjects were determined using automatic analysis. Regression and correlation analysis produced a graph where y=0.207x + 0.131, and a correlation coefficient of r=0.6228 (Fig. 5-A). The SM and PC concentrations varied widely between individuals, which is consistent with the moderate correlation. These results reflect that SM and PC play a variety of functions in plasma and cell membranes, and that plasma levels are affected by a variety of lipid metabolism pathways. Lyso-PC and PC serum levels for 50 healthy subjects were determined using automatic analysis. Regression and correlation analysis produced a graph where y=0.521x –0.4384, and a correlation coefficient of r=0.7806 (Fig. 5).

Thus, there was a relatively good correlation between lyso-PC and PC serum concentrations.

DISCUSSION

The present report describes a simple, accurate, reproducible, and rapid enzymatic method for determining SM, PC, and lyso-PC levels in serum and in lipid extracts. The method involved the use of SM- or PC-specific hydrolases, and reagents for oxidative condensation. Lyso-PC levels were estimated by subtracting the concentrations of SM and PC from the concentration of the total choline-containing phospholipids (lyso-PC = total PL – SM –PC). The enzymatic method could be adapted for both microplate and automatic analyzer analysis.

Measurements of SM and PC using the enzymatic method were found to be highly specific, sensitive, and precise, and the measurement procedures were simple and rapid. The standard methods of specific measurement and separation of SM and PC from phospholipids are thin layer gels [20] and capillary tube silica gel chromatography [21]. These methods involve separating SM and PC from crude lipids in organic solvent prior

to measurement. While HPLC is also used as a specific and sensitive method for phospholipid analysis, it requires fluorescent labeling of phospholipids [22,23].

The present enzymatic method was found to be suitable for analysis of both extracted lipids and serum lipids. Furthermore, the automatic analyzer method was rapid and very accurate. The microplate method is useful for measuring SM and PC in small samples, such as those generated following organic lipid extraction in the laboratory, while the automatic analyzer method is useful for analyzing clinical serum samples.

The present enzymatic method of measuring total PL, SM, and PC was characterized by specific measurement of the choline base of phospholipids, and the accuracy of measurement of the three lipid species was excellent. SM, PC, and lyso-PC in plasma and cell membranes accounts for most of the total choline-containing phospholipids, and the amounts of lyso-SM are relatively small. Therefore, the lyso-PC levels could be estimated using the following calculation: lyso-PC = total PL–(SM + PC). Measurement of lyso-PC can be used to estimate phospholipase A2 (PLA2) activity in PC metabolism and also the oxidative effects of phospholipids since the lyso-PC in plasma LDL is associated with LDL oxidation [24,25].

In clinical laboratory tests, the total PL level in serum is generally used as an index of

hepatic disorders and hepatobiliary disease, and the fractional abnormality of serum phospholipids is a marker for hepatic cirrhosis and obstructive jaundice [26,27]. Recently, it has been reported that phospholipids and phospholipid hydrolysates play important roles in bioactivity as lipid mediators and in the development of atherosclerosis from hyperlipemia [28,29]. While it is important to determine the clinical levels of phospholipids, the standard measuring methods involving TLC or capillary silica gel chromatography are neither simple nor quantitative. The levels of SM, PC, and lyso-PC and the SM/PC ratio in serum and cells determined using the present enzymatic method were consistent with those determined using standard methods. We have previously reported on the use of TOF-MS to analyze the fatty acid composition of phospholipids [17]. Variations in the fatty acid composition of serum or cell membrane phospholipids can be determined using a combination of TOF-MS analysis and the present quantitative enzymatic method.

In conclusion, the present enzymatic method for direct serum SM and PC measurement is rapid, specific and sensitive. The concentration of lyso-PC can be estimated by subtracting the SM and PC concentrations from the concentration of total choline-containing phospholipids. The microplate approach allows for analysis of small

samples (e.g., lipid extracts from plasma lipoproteins, cells and tissues), while the automatic analyzer approach allows for analysis of larger volumes (e.g., clinical samples).

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FIGURE LEGENDS

Fig. 1. Assay procedure to determine sphingomyelin (A) and phosphatidylcholine (B) concentrations using the microplate and automatic analyzer methods.

Fig. 2. Mass spectrometry analysis of the hydrolysis of HDL-phospholipids with sphingomyelinase or PC-specific phospholipase D. Human serum high density lipoprotein (HDL) were incubated without (A) or with sphingomyelinase (SMase; B) or PC-specific phospholipase D (PLD; C) solutions at 37°C for 30 min, and subjected to MALDI-TOF-MS analysis as described at the method section.

Fig. 3. Calibration of the microplate and automatic analyzer methods. Standard solutions of SM (open circles) and PC (closed circles) were analyzed using the microplate (A) and automatic analyzer (C) methods. Human serum was serially diluted with saline, and SM (open circles) and PC (closed circles) levels were determined using the microplate (B) and automatic analyzer (D) methods.

Table 1. Reproducibility of serum SM, PC, and lyso-PC measurements using the microplate and automatic analyzer methods.

Fig. 4 Relationship between human serum SM, PC, and total choline-containing phospholipid (total PL) levels.

Fig. 5 Correlations between serum PC, SM, and lyso-PC levels. A: Correlation between PC and SM. B: Correlation between PC and lyso-PC.

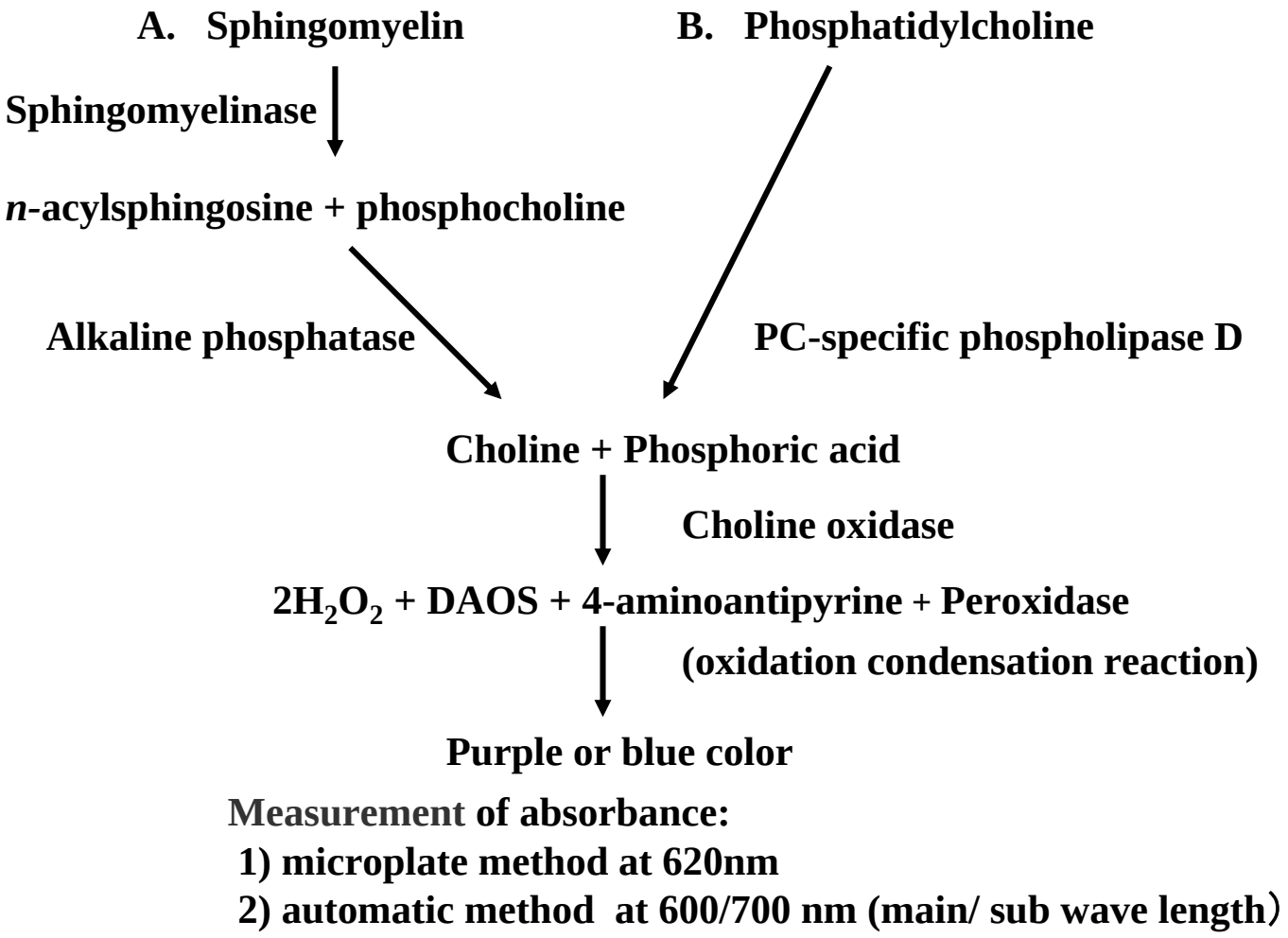

A. Sphingomyelin
B. Phosphatidylcholine
Sphingomyelinase
n-acylsphingosine + phosphocholine
Alkaline phosphatase
PC-specific phospholipase D
Choline + Phosphoric acid
Choline oxidase
2H2O2 + DAOS + 4-aminoantipyrine + Peroxidase
(oxidation condensation reaction)
Purple or blue color
Measurement of absorbance:
1) microplate method at 620nm
2) automatic method at 600/700 nm (main/ sub wave length）

Fig.1

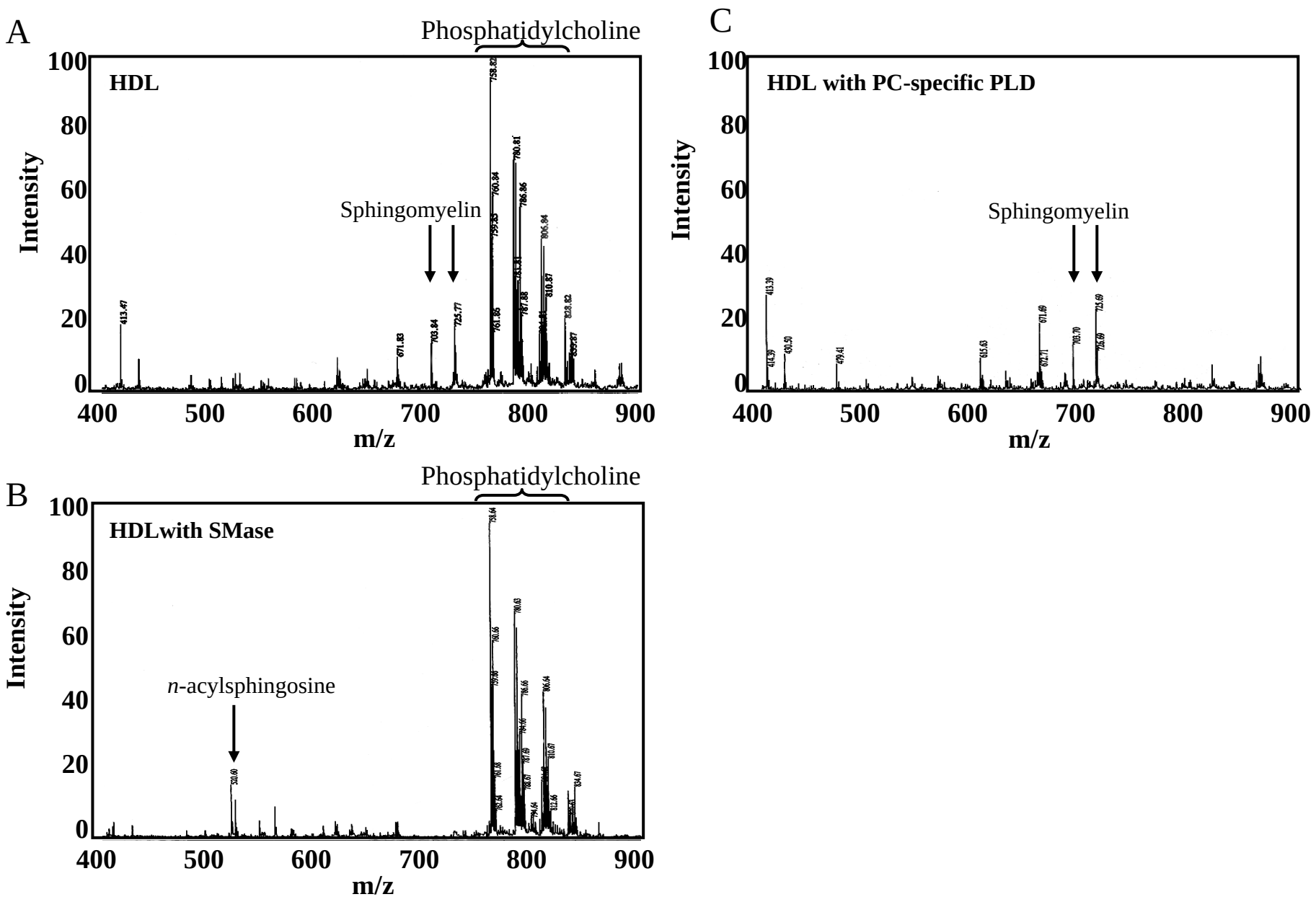

A
Phosphatidylcholine
HDL
Sphingomyelin
Intensity
m/z
B
Phosphatidylcholine
HDLwith SMase
n-acylsphingosine
Intensity
m/z
C
HDL with PC-specific PLD
Sphingomyelin
Intensity
m/z

Fig.2

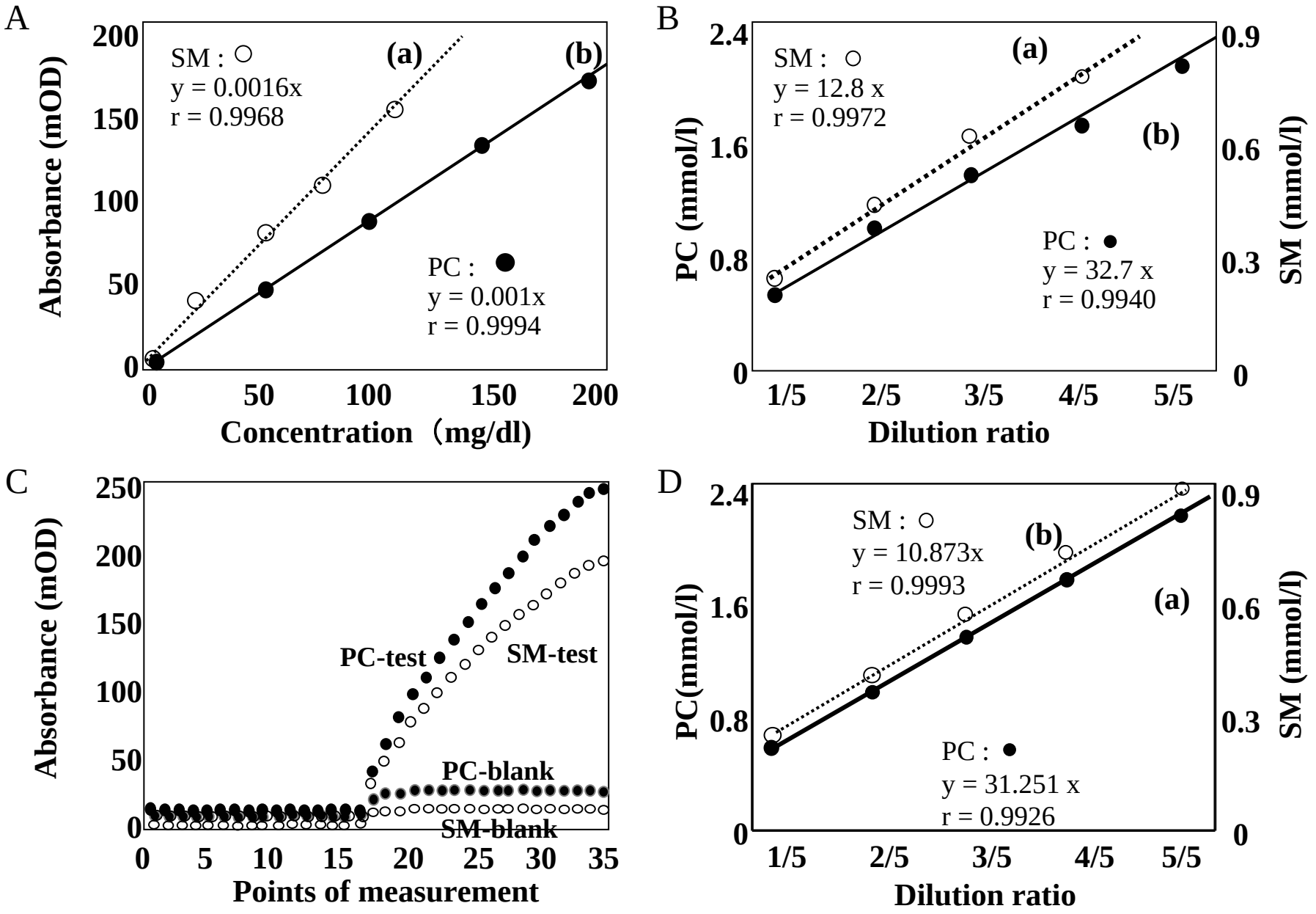

A
Absorbance (mOD)
Concentration（mg/dl)
SM : ○
y = 0.0016x
r = 0.9968
PC : ●
y = 0.001x
r = 0.9994
(a)
(b)
B
PC (mmol/l)
SM (mmol/l)
Dilution ratio
SM : ○
y = 12.8 x
r = 0.9972
PC : ●
y = 32.7 x
r = 0.9940
C
Absorbance (mOD)
Points of measurement
PC-test
SM-test
PC-blank
SM-blank
D
PC(mmol/l)
SM (mmol/l)
Dilution ratio
SM : ○
y = 10.873x
r = 0.9993
PC : ●
y = 31.251 x
r = 0.9926

Fig. 3

Table 1 Reproduciblity of serum SM and PC measurements

	Microplate						Autoanalyzer					
	SM		PC		Lyso-PC		SM		PC		Lyso-PC	
Sample	Mean (SD)	CV	Mean (SD)	CV	Mean (SD)	CV	Mean (SD)	CV	Mean (SD)	CV	Mean (SD)	CV
Intraassay [a]												
I	336 (12)	3.6	1237 (43)	3.5	370 (14)	3.8	319 (5)	1.6	1210 (11)	0.9	338 (9)	2.7
II	563 (14)	2.5	2063 (53)	2.5	599 (14)	2.3	550 (7)	1.3	1971 (18)	0.9	551 (9)	1.6
III	701 (16)	2.3	2476 (51)	2.0	718 (16)	2.2	684 (7)	1.0	2374 (21)	0.9	698 (10)	1.4
Interassay [b]												
I	340 (14)	4.1	1226 (46)	3.7	364 (16)	4.4	310 (7)	2.2	1207 (13)	1.1	340 (10)	2.9
II	556 (17)	3.0	2046 (58)	2.8	592 (16)	2.7	555 (9)	1.6	1993 (21)	1.0	563 (11)	1.9
III	698 (17)	2.4	2464 (59)	2.4	710 (17)	2.4	687 (10)	1.4	2392 (24)	1.0	702 (13)	1.8

Unit for Mean and SD are umol/l, and for CV are %.

[a] Twenty aliquots of each serum sample were assayed simultaneously.

[b] The three sera were assayed in duplicate each day for 10 days using the same reagent lot.

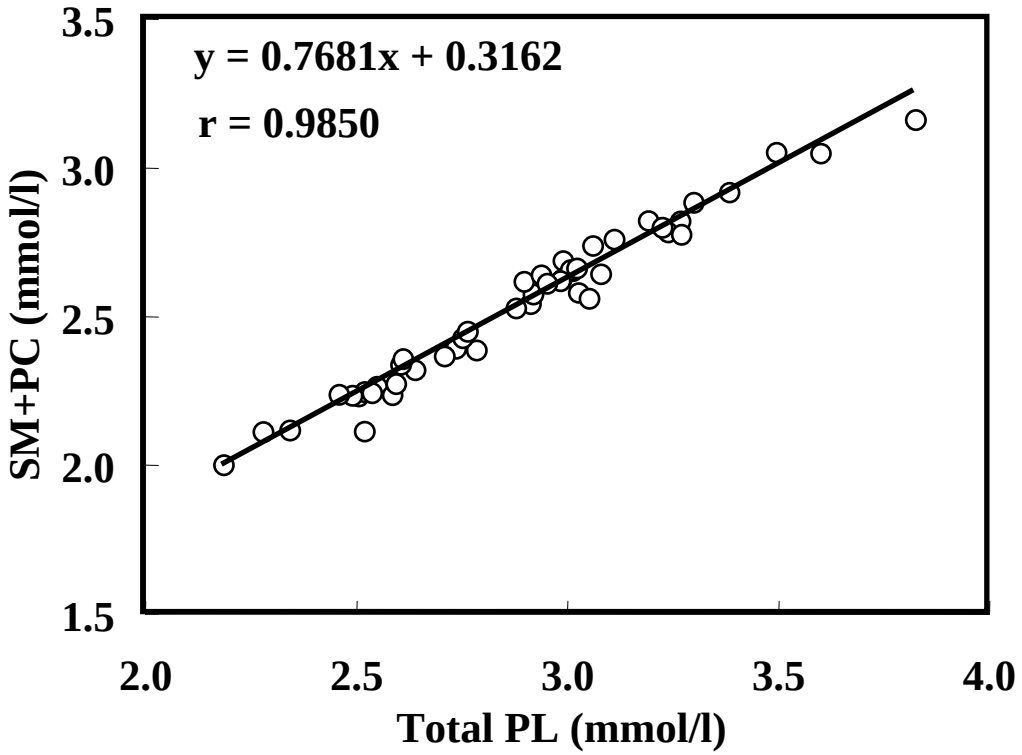
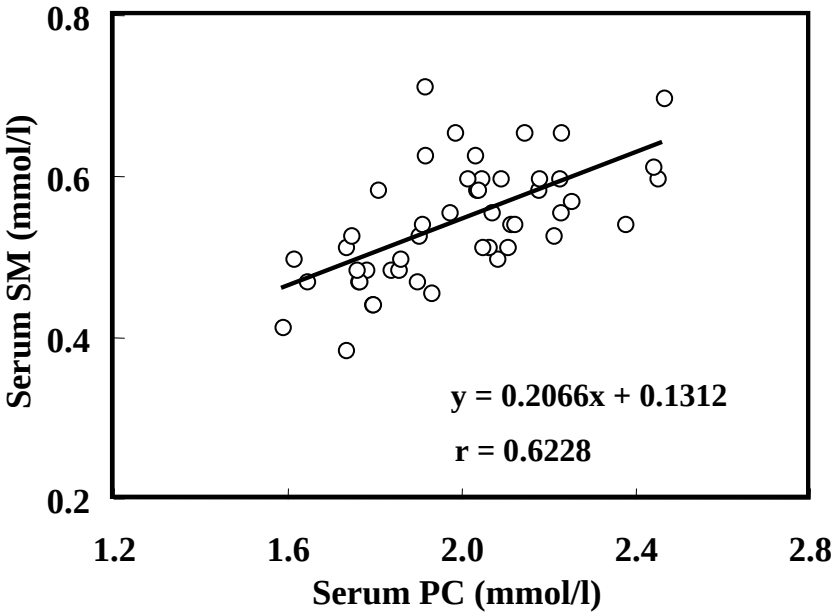

y = 0.7681x + 0.3162
r = 0.9850
SM+PC (mmol/l)
Total PL (mmol/l)
3.5
3.0
2.5
2.0
1.5
2.0
2.5
3.0
3.5
4.0

Fig.4

A


0.8
0.6
0.4
0.2
Serum SM (mmol/l)
y = 0.2066x + 0.1312
r = 0.6228
1.2
1.6
2.0
2.4
2.8
Serum PC (mmol/l)

B

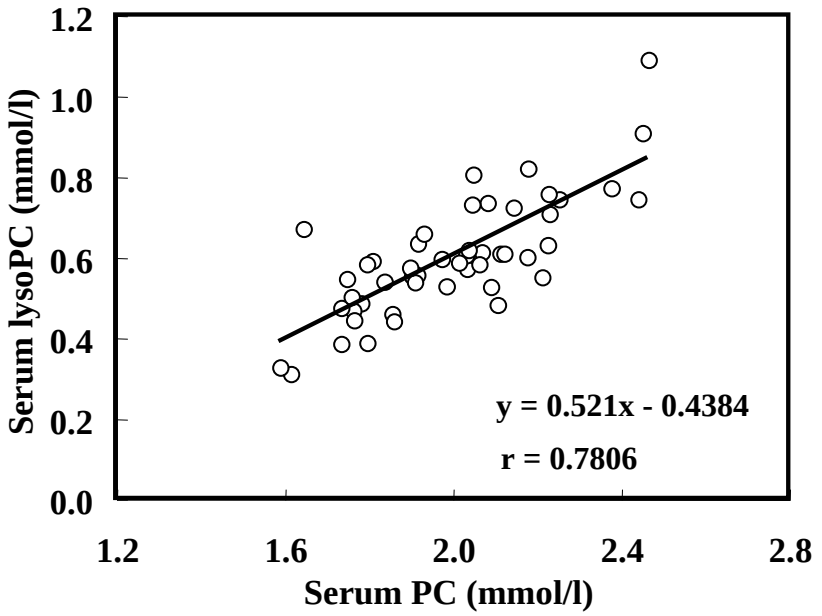

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Serum lysoPC (mmol/l)
y = 0.521x - 0.4384
r = 0.7806
1.2
1.6
2.0
2.4
2.8
Serum PC (mmol/l)

Fig.5