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Sensitive flow-injection method with peroxyoxalate chemiluminescence detection combined with preparative high-performance liquid chromatography for determination of choline-containing phospholipids in human serum

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Abstract

A sensitive and rapid flow-injection analysis (FIA) of total choline-containing phospholipids (PLs) and a selective FIA method for the class assay of choline-containing PLs combined with preparative HPLC were described. The FIA method is based on peroxyoxalate chemiluminescence (PO-CL) detection of hydrogen peroxide enzymatically formed from choline-containing PL. The linear standard curves were obtained up to 1 nmol/20-μl injection (r>0.999) with the detection limits of 1.3–1.6 pmol at a signal-to-noise ratio of 2. The total amounts of choline-containing PLs in human serum were ranged from 1.63 to 3.19 mg/ml. The HPLC separation of choline-containing PLs was achieved with an aminopropyl-modified silica gel column using a mixture of acetonitrile–methanol–10 m*M* ammonium phosphate buffer pH 5.8 as eluent. The eluate corresponding to each choline-containing PL was collected, evaporated, dissolved in 0.1% Triton X-100 aqueous solution, and then injected into FIA system. The FIA method combined with preparative HPLC was applied to the assay of human serum.

Keywords: Peroxyoxalate chemiluminescence detection; Phospholipids; Choline

1. Introduction

Phospholipids (PLs) are very important and major blood serum lipids, and their quantities in the clinical analysis are used for diagnosis of liver disease such as obstructive jaundice [1]. The PL composition of pulmonary surfactant in general consists of seven major PL classes and has been discussed on its importance in maintaining the functional characteristic of surfactant [2,3]. Further, PL vesicles have been used as pharmaceutical carriers [4]. Thus, sensitive and simple determination methods of PLs are requisite for these studies, and many methods have been developed; HPLC with ultraviolet (UV) detection [3,5,6], refractive index (RI) detection [4], chemiluminescence detection [7], evaporative laser light-

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scattering detection [8,9], colorimetric detection of hydrogen peroxide enzymatically formed from choline-containing PLs [10], and flow-injection analysis (FIA) with chemiluminescence (CL) detection [11]. Among these, UV detection is very simple and convenient, and has been most commonly used. However, the UV response is highly dependent on the nature of fatty acid residue of PLs and varies with the degree of unsaturation. RI method is relatively insensitive. CL detection with luminol as a chemiluminescent reagent is the most sensitive for choline-containing PLs, and thus suitable for the assay of micro amounts of sample.

Peroxyoxalate-chemiluminescence (PO-CL) method has been used for the highly sensitive quantification of hydrogen peroxide and fluorescent compounds. Previously, we developed the rapid and sensitive FIA method with PO-CL detection of uric acid and glucose using immobilized enzyme column reactor (IMER) [12] and the highly sensitive and selective HPLC–PO-CL method of hydrogen peroxide [13].

In this paper, we studied a sensitive and rapid FIA–PO-CL method for the determination of total amounts of choline-containing PLs using a column reactor immobilized with phospholipase D (PLD) and choline oxidase (ChO). For the determination of individual choline-containing PL, HPLC separation of the PLs was achieved prior to the FIA. The FIA method combined with preparative HPLC was applied to the assay of choline-containing PLs in human serum.

2. Experimental

2.1. Chemicals

Bis(2,4,6-trichlorophenyl)oxalate (TCPO) and imidazole were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Phosphatidylcholine (PC), lysophosphatidylcholine (LPC), sphingomyelin (SPM), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), choline oxidase (ChO, EC 1.1.3.17) from *Alcaligenes species* with 12 U/mg and phospholipase D (PLD, EC 3.1.4.4) from *Streptomyces chromofuscus* with 2200 U/mg were obtained from Sigma (St. Louis, MO, USA). Triton X-100 and glutaraldehyde were obtained from Wako (Osaka, Japan) and platelet-activating factor (PAF) from Funakoshi (Tokyo, Japan). 2,4,6,8-Tetrathiomorpholinopyrimido[5,4,-*d*]pyrimidine (TMP) was prepared by our previous method [14]. Aminopropyl-controlled pore glass (particle size, 125–177 mm; pore diameter, 500 Å) from Pierce (Rockford, IL, USA) was used for immobilizing enzymes. Water was deionized and passed through the Pure Line WL 21 P system (Yamato Scientific, Tokyo, Japan). Acetonitrile and methanol used were of HPLC grade (Wako). The other chemicals used were of analytical reagent grade.

2.2. Preparation of immobilized enzyme column reactor

The IMER was prepared with glutaraldehyde method [15] as follows; a suspension of 100 mg aminopropyl-controlled pore glass in 5 ml of 2.5% glutaraldehyde aqueous solution was stirred for 1 h at room temperature. After the glass beads were filtered and rinsed with 50 m*M* phosphate buffer (pH 8.0), the beads were suspended in 1 ml of the same buffer containing 2.5 mg each of PLD and ChO. After standing for 12 h at 4°C, the beads were washed with the same buffer solution and packed in a small stainless-steel column (70 × 2 mm I.D.) with suction. The IMER thus prepared and stored in a refrigerator in 50 m*M* phosphate buffer pH 8.0, was able to be used at least 2000 shots.

2.3. FIA–PO-CL system

A flow diagram for the measurement of CL is shown in Fig. 1. It consists of two high-performance liquid chromatographic pumps (LC-6A, Shimadzu, Kyoto, Japan), a 7125 injector with a 20-μl sample loop (Rheodyne, Cotati, CA, USA), a chemiluminescence detector (CLD-10A, Shimadzu), a signal cleaner (UN-1, Union, Gunma, Japan), and a recorder (FBR-1, Tosoh, Tokyo, Japan). 10 m*M* Imidazole buffer (pH 8.0) containing 0.1% Triton X-100 as a carrier solution and a mixture of 0.4 m*M* TCPO and 0.5 μ*M* TMP in acetonitrile as a post column CL reagent were used. The flow-rates of the carrier solution and the CL reagent were set at 1.0 and 1.2 ml/min, respectively.

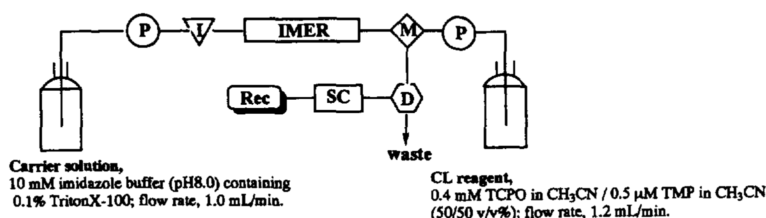

Fig. 1. Flow-injection system for the measurement of chemiluminescence. P, pump; I, injector with 20-μl loop; IMER, immobilized enzyme reactor (70 × 2 mm I.D.); M, mixing tee; D, detector; SC, signal cleaner; Rec, recorder.

2.4. Colorimetric determination of total choline-containing phospholipids

The total amounts of choline-containing PLs in human serum were measured by using a commercially available kit (Phospholipid B-Test Wako) to compare those obtained by the FIA method developed. To 3 ml of the reagent solution (0.36 U/ml PLD, 1.7 U/ml ChO, 4.3 U/ml peroxidase, 0.74 m*M* 4-aminoantipyrine in 45 ml of 50 m*M* Tris buffer (pH 8.0) containing 5.3 m*M* phenol) was added 20 μl of a standard mixture of choline-containing PLs or serum. The mixture was incubated at 37°C for 10 min. The absorbance of the mixture was measured at 505 nm against the reagent blank.

2.5. Preparation and determination of serum sample

Serum samples were obtained from healthy volunteers in our department. For the measurement of total choline-containing PLs, serum (5 μl) was diluted to 2 ml with 0.1% Triton X-100 aqueous solution, and then, 20 μl-aliquot of the resultant was injected into FIA system.

The working curve was prepared as follows; to the residue obtained after evaporating 100 μl of a known concentration of standard choline-containing PL mixture were added 5 μl serum and 1995 μl 0.1% Triton X-100 aqueous solution and the solution was vortex-mixed for 1 min. An aliquot of the resultant mixture (20 μl) was injected into FIA system. For a blank, chloroform–methanol (50:50, v/v) was used instead of a standard solution and the blank was treated as described above. The standard mixture of PLs were prepared by mixing 4 m*M* each of PC, SPM and LPC in chloroform–methanol (50:50, v/v) in a ratio of 70:20:10 (v/v/v). The molecular masses for PC, SPM and LPC, 790.2, 749.1 and 523.7, respectively, were used tentatively, because the correct formulas for these PLs were unknown.

For the class assay of choline-containing PL, serum was treated as in Fig. 2. To 5-μl serum was added 100 μl of chloroform–methanol (50:50, v/v) and the solution was passed through a membrane filter (0.45 μm, Ekikurodisk, Gelman Sciences Japan, Tokyo). A mixture of chloroform–methanol was used to extract the PLs effectively. A 20-μl aliquot of the filtrate was injected into the HPLC system equipped with LC-6A pump (Shimadzu), UVIDEC-100-IV UV-detector (Jasco, Tokyo, Japan), and an aminopropyl-modified silica gel column (Daisopak-SP-120-5-APS, 5 μm, 150 × 6.0 mm I.D., Daiso, Osaka, Japan) using a mixture of

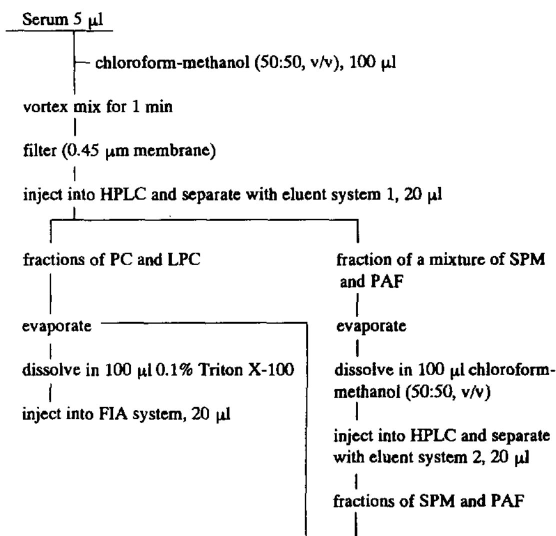

Fig. 2. Assay procedure for serum sample.

acetonitrile–methanol–10 m*M* ammonium phosphate buffer pH 5.8 (61.5:26.4:15, v/v/v) as an eluent system 1 at a flow-rate of 1 ml/min. An injection of chloroform–methanol on the APS column gave no remarkable change in the HPLC results. The fractions corresponding to choline-containing PLs were collected and evaporated with a centrifugal evaporator (RD-31, Yamato Scientific, Tokyo, Japan), and the residues for PC and LPC were dissolved in 100 μl of 0.1% Triton X-100. A 20-μl aliquot of the resultant solution was injected into FIA system. While, the fraction corresponding to a mixture of SPM and PAF was evaporated and dissolved in 100 μl of chloroform–methanol (50:50, v/v), and then, 20 μl of the resultant solution was injected into HPLC using acetonitrile–methanol–water (61.6:26.4:10, v/v/v) containing 0.1% sodium octanesulfonate as an eluent system 2 at a flow-rate of 1 ml/min. The eluates were monitored at 205 nm. The eluate corresponding to each PL was collected and evaporated to dryness. The resultant residue was dissolved in 100 μl of 0.1% Triton X-100 solution and a 20-μl aliquot of the mixture was injected into the FIA system.

3. Results and discussion

3.1. FIA of total choline-containing phospholipids

CL reaction conditions were first examined by using the FIA system in Fig. 1 without the IMER. As shown in Fig. 3, the CL intensity increased with an increase in concentration of both TCPO and TMP; 0.4 μM TCPO and 0.5 m*M* TMP were selected because the largest signal-to-noise ratios were obtained at these concentrations, respectively. The optimal pHs for the enzymatic reactions of both ChO from *Alcaligenes species* and PLD from *Streptomyces chromofuscus* are known to be 8.0, and thus, we used imidazole buffer of pH 8.0 as a carrier solution in the succeeding experiments.The effect of reagent concentrations on CL intensity was examined with standard solutions of 10 and 20 pmol on column of PC. The largest CL intensity was obtained with 10 m*M* imidazole buffer (Fig. 4A). Triton X-100 has been reported to enhance TCPO CL intensity [16], and, thus, the effect of Triton X-100 on CL was examined. As shown in Fig. 4B, the maximum and constant intensities were obtained over the range from 0.05–0.1%; 0.1% Triton X-100 was used in this study. The role of Triton X-100 in CL reaction has not been elucidated; it may increase the efficiency of energy transfer from the reaction intermediate of TCPO and hydrogen peroxide, e.g., 1,2-dioxetane-3,4-dione, to fluorophore, and, as a result, the CL intensity may increase.

The linear relationships were obtained up to 1 nmol/20-μl injection between the recorder responses and the concentrations of PC, LPC, SPM and PAF (r>0.999). The detection limits of PC, LPC, SPM and PAF at a signal-to-noise ratio (S/N) of 2 were 1.5, 1.3, 1.5 and 1.6 pmol/injection,

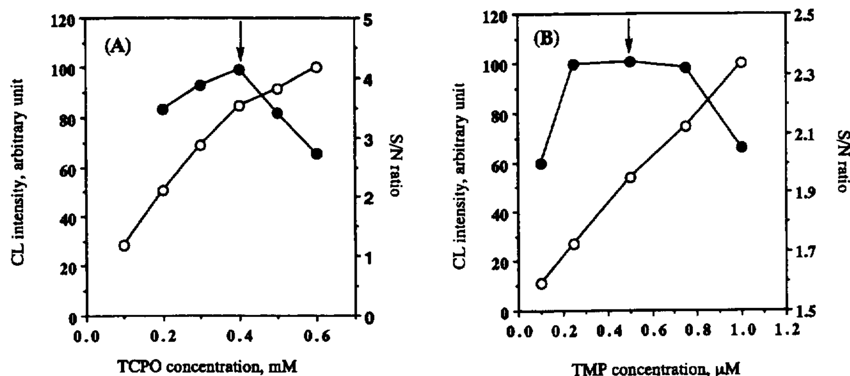

Fig. 3. Effect of the concentration of (A) TCPO and (B) TMP on chemiluminescence intensity. (A) (○) H_2O_2 at 20 pmol/injection, (●) S/N ratio; 20 m*M* imidazole buffer (pH 7.0), 0.5 μM TMP. (B) (○) H_2O_2 at 20 pmol/injection, (●) S/N ratio; 20 m*M* imidazole buffer (pH 7.0), 0.4 m*M* TCPO.

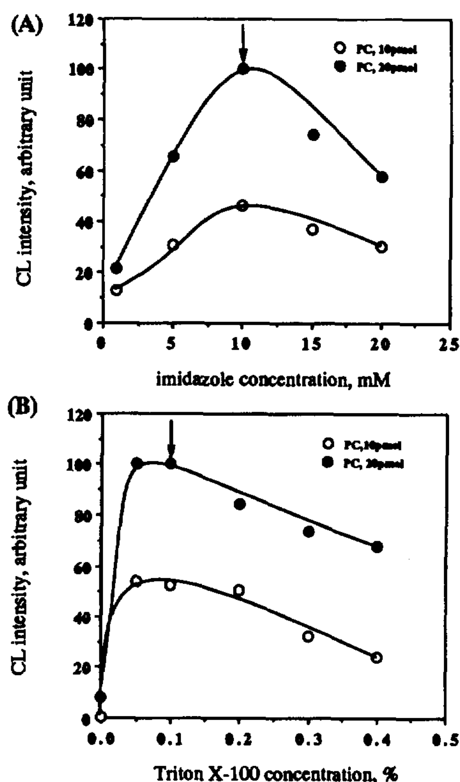

Fig. 4. Effect of the concentration of (A) imidazole and (B) Triton X-100 on chemiluminescence intensity. PC concentration: (○) 10 pmol/injection, (●) 20 pmol/injection; CL reagent: 0.4 m*M* TCPO, 0.5 μM TMP; (A) imidazole buffer (pH 8.0), 0.1% Triton X-100. (B) 10 m*M* imidazole buffer (pH 8.0).

respectively. The relative standard deviations (R.S.D.) for the measurements of seven samples (200 pmol per injection) were in the range of 1.6–3.3%.

The proposed method was applied to the assay of total choline-containing PLs in human serum. The working curve was prepared using a spiked serum with a known concentration of standard mixture of PLs (PC–SPM–LPC=70:20:10, v/v/v). The linear relationship was obtained up to 6.0 mg/ml of serum (Fig. 5). The R.S.D. for within-day and between-day assay of serum sample containing 3.12 mg/ml were 1.7% (n=7) and 7.7% (n=5), respectively. The recovery calculated from the slopes of standard graphs of standard solution and spiked serum was 94%. The total amounts of choline-containing PLs obtained for normal serum samples were ranged

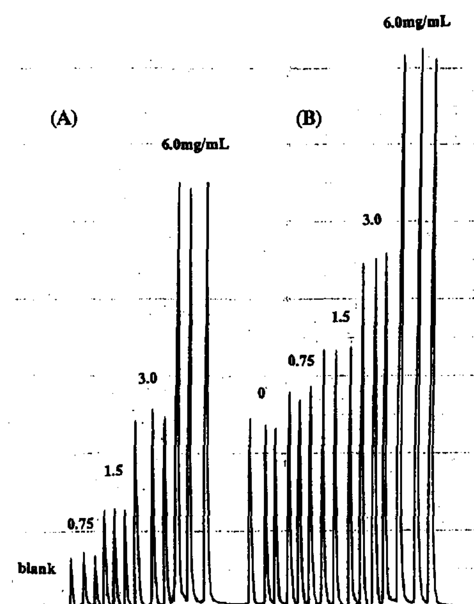

Fig. 5. Recorder responses of (A) a standard mixture of choline-containing phospholipids and (B) spiked serum. FIA conditions are same as in Section 2. Figures on the peaks are the concentrations of phospholipids in a standard mixture or spiked in serum.

from 1.63 to 3.19 mg/ml. The results obtained by the proposed method were compared to those obtained with the colorimetric method (Fig. 6). The linear regression and correlation coefficient between the present method (x) and the colorimetry (y) were y=0.87x − 0.16 (r=0.936, n=14). The slightly

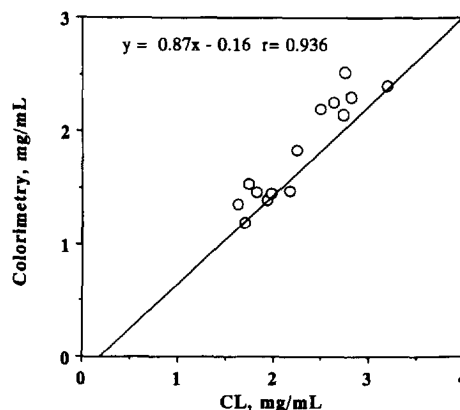

Fig. 6. Correlation between the amounts of total choline-containing phospholipids by the proposed method and the conventional colorimetric method (n=14).

smaller correlation coefficient might be caused by the narrow total choline-containing PL concentration ranges of normal sera used. By measuring a variety of serum samples containing wide PL concentration ranges, the correlation should become better.

3.2. HPLC–FIA assay of phospholipids

A number of methods have been used for the separation of PLs [3–8]. Among these, some complex gradient systems were used for the separation of choline-containing PLs from other lipids. On the other hand, Shimbo showed a good isocratic separation of the choline-containing PLs using an aminopropyl-modified silica gel [6]. Therefore, we adopted this method and attained a good separation of six kinds of phospholipids (PC, SPM, LPC, PE, PI, and PS) as shown in Fig. 7. The retention times of PC, SPM and LPC were 4.6, 5.9, and 7.8 min, respectively. Under the conditions, the peak of SPM was unfortunately overlapped with that of PAF. Thus, we had to use additional separation conditions. As shown in Fig. 8, SPM and PAF could be separated using eluent system 2. The retention times for PAF and SPM were 9.9 and 13.1 min, respectively. After separation, the eluates were dried up and dissolved in 0.1% Triton X-100. The resultant solution was injected into the FIA system.

The good linearities between the recorder responses and the concentrations of phospholipids were obtained (>0.998) over the range of 10–1000 pmol/injection with the detection limits ($S/N=2$) of 1.6 (PC), 2.0 (LPC), 2.1 (SPM), and 2.2 pmol (PAF). The sensitivity of the method was 100–3000 times higher than those with UV detection [3,5]. The HPLC–FIA method was applied to the assay of choline-containing phospholipids in serum. Good linearities for PC, LPC, SPM and PAF were obtained over the range from 50–1000 (0.197–3.952), 25–600 (0.0637–1.529), 25–400 (0.093–1.488), and 12.5–600 pmol/injection (0.0336–1.613 mg/ml

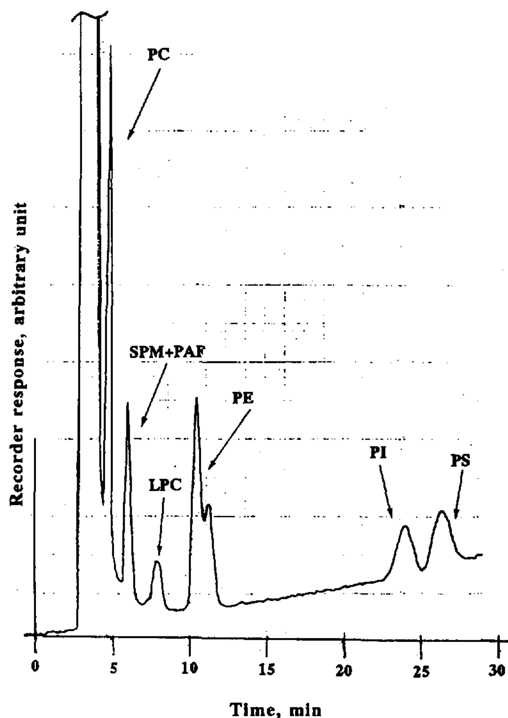

Fig. 7. Chromatogram of the standard phospholipids. Sample: PC, SPM, PE, PI, PS (2 nmol/injection), LPC and PAF (20 nmol/injection). Separation was achieved with eluent 1; other HPLC conditions are as in Section 2.

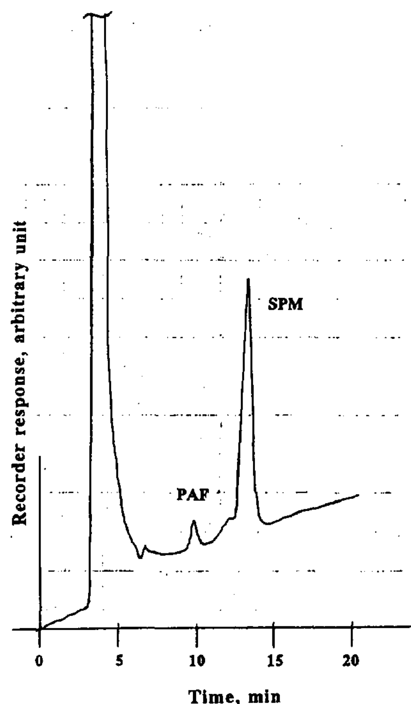

Fig. 8. Chromatogram of SPM and PAF. Sample: SPM (2 nmol/injection) and PAF (20 nmol/injection). Separation was achieved with eluent 2; other HPLC conditions are as in Section 2.

Table 1
Precision and accuracy of FIA–HPLC method

Compound	Nominal concentration (mg/ml)	Measured concentration (mean ± S.D., n=4) (mg/ml)	Precision (R.S.D.%)	Accuracy (mean error, %)
PC	0.790	0.751 ± 0.056	7.1	−4.9
LPC	0.510	0.505 ± 0.037	7.3	−1.0
SPM	0.744	0.625 ± 0.078	10.5	−16.0
PAF	0.538	0.524 ± 0.036	6.7	−2.6

of serum), respectively, with the regression coefficient of > 0.989. The detection limits (S/N=2) were 5.9 (PC), 4.3 (LPC), 3.0 (SPM) and 4.3 pmol per injection (PAF). Precision and accuracy of the method were determined by analyzing replicate serum samples (n=4) spiked with choline-containing PLs and PAF. The R.S.Ds of precision and the mean errors were 6.7–10.5% and (−1.0)–(−16.0)%, respectively (Table 1). The representative chromatograms of normal and spiked sera were shown in Fig. 9. The recoveries calculated from the slopes of standard graphs obtained by a standard solution and a spiked serum were 91 (PC), 95 (LPC), 83 (SPM) and 115% (PAF). The amounts of PC, LPC and SPM in serum were 1.634 ± 0.38, 0.166 ± 0.057 and 0.485 ±0.176 mg/ml (n=20), respectively; the composition ratio was 71.9:7.3:20.7(%) which corresponded well with those reported [17]. On the other hand, PAF could not be detected under the conditions used. Because

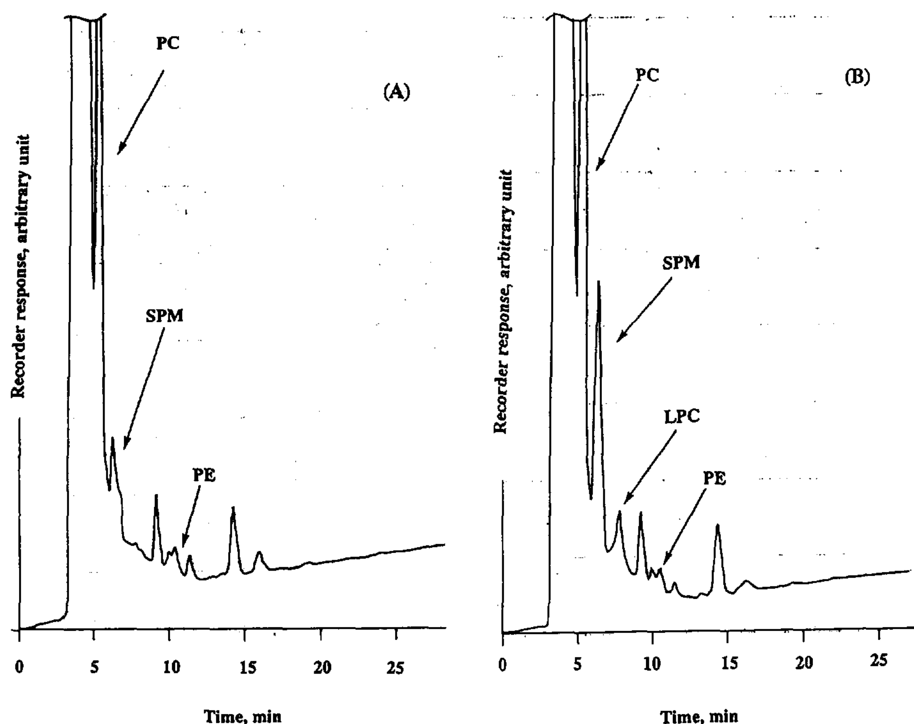

Fig. 9. Chromatograms of normal and spiked sera. Sample: (A) normal serum; (B) serum spiked with PC, SPM (2 nmol/injection) and LPC (20 nmol/injection). Separation was achieved with eluent 1; other HPLC conditions are as in Section 2.

Table 2
Variance of HPLC–FIA assay of choline-containing phospholipids in serum

Compound	Within-day (n=5)		Between-day (n=3)	
	Mean ± S.D. (mg/ml)	R.S.D. (%)	Mean ± S.D. (mg/ml)	R.S.D (%)
PC	2.26 ± 0.123	5.4	1.77 ± 0.175	9.9
LPC	0.126 ± 0.014	11.0	0.143 ± 0.013	9.0
SPM	0.681 ± 0.048	7.0	0.701 ± 0.030	4.3

the sensitivity of the proposed method was not enough to determine the very low levels of PAF in whole blood (10–30 pg/ml) [18] and plasma (54 ± 40 pg/ml) [19]. The R.S.D.s. for within-day assay (n=5) and between-day assay for a week (n=3) were smaller than 11% as shown in Table 2.

4. Conclusion

A simple and sensitive FIA method with PO-CL detection was developed for the determination of total choline-containing PLs in serum. The method only needs a dilution of serum with 0.1% Triton X-100 aqueous solution prior to analysis and the sensitivity was comparable to those by other CL methods [7,11]. Further the class assay of choline containing PLs could be achieved by combination of above FIA method with preparative HPLC. The sensitivity of this combined FIA–HPLC method is 100–3000 times higher than those of HPLC–UV methods. The proposed methods might be useful for the studies on choline-containing phospholipids in a variety of biological samples.

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