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Simultaneous determination and quantification of seven major phospholipid classes in human blood using normal-phase liquid chromatography coupled with electrospray mass spectrometry and the application in diabetes nephropathy

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ABSTRACT

A rapid and specific analytical method for simultaneous determination and quantification of seven major phospholipid classes in human blood was developed by normal-phase high-performance liquid chromatography tandem mass spectrometry. The optimal separation was achieved by using mobile phase hexane (A) and 2-propanol with water, formic acid and ammonia as modifiers (B) using an HPLC diol column. Isocratic elution method was used for better repeatability and no balance time. The seven major phospholipid classes in human blood that were detected including phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI) phosphatidylcholine (PC), lysophosphatidylcholine (Lyso-PC), and sphingomyelin (SM). That can be separated in this condition. Every phospholipid class contains many molecular species which have similar structure. The structure of phospholipids molecular species was identified by ion-trap MS^n which produced ion fragments. And the qualification was completed by TOF-MS which shows good accuracy. Through the accurate quantification of one representative phospholipids molecule in each class, a method for simultaneous estimation hundreds of molecular species in seven major classes was established. The intra-day and inter-day precision and recovery had been investigated in detail. The RSD of precision for most compound is below 8% and RE is below 10%. Recovery is almost over 80%. This method was applied to phospholipids disorder related with diabetes nephropathy successfully. The concentrations of most phospholipids for normal people are higher than that for diabetic nephropathy (DN) patients in three phases. For most of phospholipids, with the development of DN the concentration was decreasing.

1. Introduction

Phospholipids consist of a polar head group (HG) with a phosphate moiety and two fatty acids (FAs) that are attached to the glycerol backbone. Based on differences in their polar head groups, the phospholipids are divided into several classes. Each class of phospholipids is composed of a mixture of many molecular species containing different fatty acids. So there are numerous phospholipids species.

Phospholipids are the main constituents of biological membranes and have important structural and functional properties. Both the physical and chemical properties of the membrane bilayer can be affected by the variation of phospholipids compositions. Some phospholipids also participate in biological processes in various ways [1,2]. Some studies have demonstrated that diabetes mellitus is associated with metabolism disorder of lipid or fatty acid in phospholipids [3–6]. Diabetic nephropathy (DN) is an important complication of both Type 2 and Type 1 diabetes [7,8]. It is the leading cause of chronic kidney disease in patients starting renal replacement therapy. In this paper researches have been done to find out the relationship between the disorder of phospholipids and DN. Phospholipids are also used in many fields such as making liposome for drug delivery and bio-membranes. So analyses of the different classes and species of phospholipids are very important both for basic disease-related research and for the pharmaceutical industry.

Both the composition and the concentration of molecular species can affect the function of membrane. Many methods have

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been developed for analysis of the molecular species in different phospholipid classes. Chromatographic techniques has been used that separate the different phospholipid classes, such as thin-layer chromatography (TLC) [9,10], gas chromatography [11] high-performance liquid chromatography (HPLC) [12,13]. Because of the polarity of the phospholipids, normal-phase HPLC system is good choice. There are several method developed for this [14–16]. In this paper an isocratic method by normal-phase HPLC was described for class separation of phospholipids in blood. Seven major phospholipid classes can be detected in this method which showed better repeatability and needed no balance time. It was proved to be a good method with more advantages for high-through analysis.

Detection of phospholipids has been performed by different spectrophotometer techniques such as UV, ELSD [16–20]. But these techniques have poor ability for identification of the different phospholipids species. Mass spectrometry (MS) has strong identification ability, high sensitivity, specificity, and simplicity so it is a very useful tool for analysis of phospholipids composition. The introduction of "soft" ionization methods has completely opened new vista in this field. In particular, electrospray ionization-mass spectrometry (ESI-MS) has been shown to be a very attractive technique [21,22]. Different MS has different advantages. Because of strong power in separation and determination, HPLC–MS is now widely used for lipid analysis.

In this paper, a sensitive qualitative and quantitative analysis method was developed by using different mass spectrometry. Ion-trap tandem mass spectrometry which can produce the product ions and fragments by breaking the mother ion into pieces was used for determining the species of phospholipids. Time of flight mass spectrometry which has higher sensitivity, specificity and exact molecular weight was used for qualification. We focus on seven major phospholipid classes in human blood [23] including phosphatidylserine (PS), phosphatidylethanolamine (PE), Phosphatidylglycerol (PG), phosphatidylinositol (PI) phosphatidylcholine (PC), lysophosphatidylcholine (Lyso-PC), and sphingomyelin (SM). Through the accurate quantification of one representative phospholipids molecular by standard compound in each class a method for simultaneous quantification hundreds of molecular species in seven major classes was established. Although the instrument response is also related with acyl chain length and the degree of acyl chain unsaturation expect for the head group. The effect diminished and nearly disappeared with progressive dilution [24]. In this paper the concentration for the phospholipids species for several classes is low, so the effect of acyl chain length and the degree of acyl chain unsaturation is not so prominent. This approach is acceptable. The quantification data are just used for comparing the differences between cases and controls, so the relative quantification data satisfied the estimation. That turns up a good method for quantifying numerous structure-similar compounds with few standard compounds. This method was subsequently applied to phospholipids disorder related with diabetes nephropathy successfully.

2. Experiment

2.1. Chemicals and reagents

1-Stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine (PE, C18:0/C20:4, liver, bovinesodium salt), L-a-phosphatidylinositol (PI, C18:0/20:4, liver, bovine-sodium salt), 1,2-distearoyl-*sn*-glycero-3-phosphocholin (PC,C18:0/18:0), 1-stearoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (PS, C18:0/C18:1, sodium salt),1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (PG, C16:0/C18:1, sodium salt), sphingomyelin (SM, dC18:1/C18:0, brain, porcine), and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Lyso-PC, 16:0) were from Avanti Polar Lipids (Alabaster, AL, USA). 2,6-Di-*tert*-butyl-4-methylphenol was from Sigma–Aldrich (St. Louis, USA). Formic acid and ammonia is analytical grade from Modern Dong Fang (Beijing, China). Hexane and 2-propanol were of HPLC grade and purchased from J.T. Baker (Philipsburg, USA). Ultra-pure water was prepared from a Milli-Q system (Millipore, Milford, MA). All other reagents and solvents were of analytical grade and obtained from Beijing Chemical Company (Beijing, China).

2.2. High-performance liquid chromatography and mass spectrometry

An Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) was used. The LC separation was performed on a diol column (Nucleosil, 100-5 OH, Germany) (250 mm × 3.0 mm, i.d., 5.0 μm, particle size). The flow rate was 0.2 ml/min. The column temperature was at 35 °C. The isocratic elution method was used for A, 30%; B, 70%. Mobile phase A: hexane; mobile phase B: 2-propanol/water/formic acid/ammonia (86/13/1.0/0.12, v/v).

The Agilent 1100 HPLC system was coupled on-line to an ion-trap mass spectrometer (Agilent Corp, Waldbronn, Germany) equipped with an electrospray ionisation (ESI) source. The operation parameters are as follows: negative-ion mode (ESI^-); nitrogen drying gas, 9 l/min; nebulizer, 35 psi; gas temperature, 350 °C; compound stability, 80%; mass range, 50–1000*m*/*z*.

The Agilent 1100 HPLC system was coupled to a TOF-MS (Agilent Corp., Waldbronn, Germany) equipped with an electrospray interface. The HPLC conditions for the HPLC/TOF-MS analysis were the same as those used in the HPLC analysis. TOF-MS analysis was performed in negative (ESI^-) ion mode under the following operation parameters: nitrogen drying gas 9 l/min; nebulizer 35 psi;

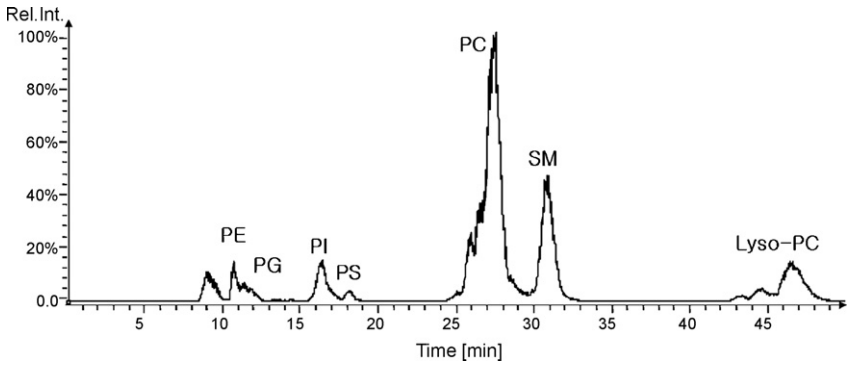

Fig. 1. Negative-ion HPLC-ESI-MS TIC spectrum from *m*/*z* 400 to 1000 of extracted human blood.

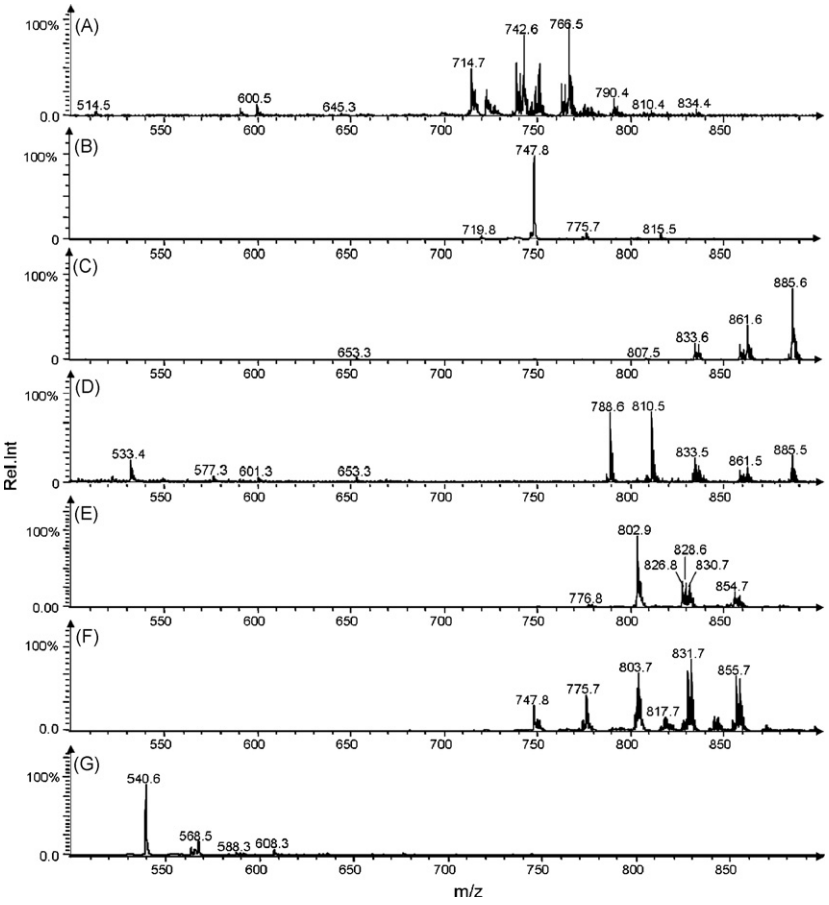

Fig. 2. Mass spectrum of phospholipids species in the seven classes shown in Fig. 1. (A) pPE/PE; (B) PG; (C) PI; (D) PS; (E) PC; (F) SM; (G) Lyso-PC.

gas temp. 350 °C. Data files were acquired in continuum (profile) mode, and spectra were stored from *m/z* 400 to 1000. Analyst QS software (Applied Biosystems, Framingham, MA) was used for data process.

2.3. Preparation of samples

Human whole blood was pooled from volunteers. The lipids in the 150 μl plasma samples were extracted essentially as described earlier [23]. Briefly, 350 μl of water was added to 150 μl plasma; then 2 ml of methanol with 0.01% (w/v) 2,6-di-*tert*-butyl-4-methylphenol and 4 ml of chloroform was added and the solution was sonicated for 60 s both before and after adding chloroform. After sonication the solution was whirlmixed for 30 s and incubated for approximately 1 h at room temperature. Finally, 2 ml of water was added before the solution was mixed for 5 s and centrifuged at 5000 rpm at 4 °C for 10 min. The lower chloroform phase was sampled and dried by evaporation under nitrogen; the samples were stored dry at −20 °C. Prior to analysis, the extracted samples were redissolved in 40 μl chloroform/methanol (2:1, v/v) and then was diluted by hexane/2-propanol (3:7, v/v) to 200 μl.

2.4. Calibration procedure

Stock solutions of 1 mg/ml PE (C18:0/C20:4), PG (C16:0/C18:1), PS (C18:0/C18:1), PI (C 18:0/20:4), PC (C 18:0/18:0), SM (dC18:1/C18:0), and Lyso-PC (C:16:0), were prepared in volumetric flasks in chloroform/methanol (2:1, v/v) individually. A series of standard working solutions with the concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 3, 4, and 5 μg/ml for PG and PS; 1, 2, 3, 4, 5, 10, 20, 30, 40, and 50 μg/ml for PE and PC; 5, 10, 20, 30, 40, 50, 60, 70, 80, and 90 μg/ml for PI, SM and Lyso-PC were obtained by further dilution with hexane/2-propanol (3:7, v/v).

Quality control (QC) samples were prepared in this way. All standard solutions were evaporated to dryness at 35 °C under a gentle stream of nitrogen. The residue reconstituted in 150 μl plasma was prepared by the same method described in Section 2.3. The concentrations of each phospholipid were at both the low and high. External calibration method was used for the quantitative analysis. Calibration curves were obtained by the plots of the peak-area versus the concentration of the calibration standards. The concentrations of the unknown samples were determined by using the equations of linear regression obtained from the calibration curves.

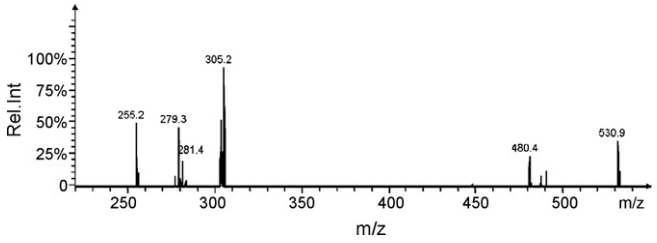

Fig. 3. MS^3 spectrum of PC species *m/z* 828.6 $[M+45]^-$.

Table 1
Identification of the major phospholipids species in human blood sample

PL	Negative-ion [M−H]-(*m*/*z*)	Combinations of molecular species		PL	Negative-ion [M−H]-(*m*/*z*)	Combinations of molecular species	
pPE/PE	778.5	pC18:0/C22:4	pC20:4/C20:4	PS	838.5	C18:0/C22:4	C20:0/C20:4
	774.5	pC18:0/C22:6	pC18:1/C22:5		836.5	C18:0/C22:5	C18:1/C22:4
	766.5	C18:0/C20:4	C16:0/C22:4		834.5	C18:0/C22:6	C18:1/C22:5
	764.5	C18:1/C20:4	C16:0/C22:5		832.5	C18:1/C22:6	C20:3/C20:4
	762.5	C16:0/C22:6			818.5	C18:0/C20:0	
	750.5	pC18:0/C20:4	pC16:0/C22:4		810.5	C18:0/C20:4	C18:1/C20:3
	746.5	pC16:0/C22:6	pC18:1/C20:5		808.5	C18:1/C20:4	C18:0/C20:5
	742.5	C18:0/C18:2	C18:1/C18:1		790.5	C18:0/C18:0	
	740.5	C18:1/C18:2	C16:0/C20:3		788.5	C18:0/C18:1	C16:0/C20:1
	738.5	C16:0/C20:4	C18:2/C18:2		786.5	C18:0/C18:2	C18:1/C18:1
	716.5	C16:0/C18:1					
	714.5	C16:0/C18:2		PI	913.5	C18:0/C22:4	C20:0/C20:4
					911.5	C18:0/C22:5	C18:1/C22:4
PG	777.5	C18:0/C18:0			909.5	C18:0/C22:6	C18:1/C22:5
	775.5	C18:0/C18:1	C16:0/C20:1		887.5	C18:0/C20:3	
	773.5	C18:0/C18:2	C18:1/C18:1		885.5	C18:0/C20:4	C18:1/C20:3
	749.5	C16:0/C18:0			883.5	C18:1/C20:4	C16:0/C22:5
	747.5	C16:0/C18:1	C18:0/C16:1		865.5	C18:0/C18:0	
	745.5	C16:0/C18:2			863.5	C18:0/C18:1	C16:0/C20:1
					861.5	C18:0/C18:2	C18:1/C18:1
SM	733.5	33:1			859.5	C16:0/C20:3	C18:1/C18:2
	745.7	34:2			857.5	C16:0/C20:4	C18:2/C18:2
	747.7	dC18:1/C16:0			837.5	C16:0/C18:0	
	775.7	dC18:1/C18:0			833.5	C16:0/C18:2	C16:1/C18:1
	801.7	C38:2					
	845.7	dC18:1/C23:0					

PL	Negative-ion [M+45]-(*m*/*z*)	Combinations of molecular species	PL	Negative-ion [M+45]-(*m*/*z*)	Combinations of molecular species	
Lyso-PC	590.3	C20:3	PC	854.5	C16:0/C22:4	C18:0/C20:4
	588.3	C20:4		850.5	C16:0/C22:6	
	568.3	C18:0		834.5	C18:0/C18:0	
	566.3	C18:1		830.5	C18:0/C18:2	C18:1/C18:1
	564.3	C18:2		828.5	C16:0/C20:3	C18:1/C18:2
	540.3	C16:0		826.5	C16:0/C20:4	C18:2/C18:2
				806.5	C16:0/C18:0	
				804.5	C16:0/C18:1	
				802.5	C16:0/C18:2	
				792.5	C16:0/C17:0	
				778.5	C16:0/C16:0	
				776.5	C16:0/C16:1	

2.5. Method validation

For precision studies, QC samples with high and low lipid concentrations were prepared in parallel. Intra-day accuracy and precision (each $n = 5$) were evaluated by analysis of QC samples at different times on the same day. Inter-day accuracy and precision (each $n = 6$) were determined by analyses of QC samples twice per day at two concentration levels over three consecutive days. The concentration of each sample was determined using calibration standards prepared on the same day. Accuracy of the method was determined by the relative error (RE), and precision was determined by the relative standard deviation (RSD). The extraction recovery was determined by comparing the peak areas obtained from QC samples subtracting blank plasma with the standard working solutions at the same concentration.

3. Results and discussion

3.1. Separation of and determination of seven phospholipid classes

Normal-phase high-performance liquid chromatography was widely used in separation of phospholipids with solvents such as chloroform, methanol, hexane, 2-propanol, and so on as mobile phase. Uran et al. [23] separated phospholipids using chloroform and methanol. But chloroform as mobile phase is dangerous for public health. Wang et al. [25] used hexane and 1-propanol which are less toxic, but the components percentage of two mobile phases is complicated in this method. The hexane is easy to volatilize, so for long time the components percentage in the mobile phase may change which will shift the retention time. We improved the method. Single solvent hexane was used as A phase and solvent mixture of 2-propanol, water, formic acid and ammonia were used as B phase. We also optimized the proportion of the mobile phase to achieve the good separation for main phospholipid classes. 1% (v/v) formic acid and 0.12% (v/v) ammonia were added in mobile phase as modifiers.

Gradient elution was used in many publications [23,25], however it required additional time to balance before the next run. We developed an isocratic elution method, which required no balance time and had a better reproducibility in retention time of chromatography than gradient elution modes we tried. Because the 2-propanol has high viscosity low flow rate 0.2 ml/min was used to avoid air bubble. Under this condition, main phospholipid classes were separated well. PE was firstly eluted, followed by PG, PI, PS, PC, SM, and Lyso-PC which are shown in Fig. 1. Because different molecular species within a class have a same polar head, their retention time in HPLC is very similar. The difference of retention time for compounds within one same class is less than that between two

Table 2
Intra-day accuracy and precision values from the assay QC standards ($n = 5$)

PL class	Low/high concentration (μg/ml)	Measured concentration mean ± RD (μg/ml)	RE (%)	RSD (%)	Extraction recoveries (%)
PE (C18:0/C20:4) $m/z = 766$	10.0	10.62 ± 0.28	6.2	2.7	88.9
	30.0	32.38 ± 2.60	7.9	8.0	93.5
PG (C16:0/C18:1) $m/z = 747$	1.0	1.09 ± 0.05	9.1	4.8	87.6
	3.0	3.13 ± 0.14	4.4	4.4	84.6
PS (C18:0/C18:1) $m/z = 788$	0.5	0.47 ± 0.03	6.0	6.8	84.4
	1.5	1.43 ± 0.11	4.7	7.6	78.8
PI (C18:0/C20:4) $m/z = 885$	20.0	20.81 ± 0.41	4.1	2.0	88.6
	50.0	52.69 ± 1.83	5.4	3.5	82.2
PC (C18:0/C18:0) $m/z = 834$	2.0	1.87 ± 0.08	−6.5	4.0	80.3
	10.0	9.13 ± 0.51	−8.7	5.6	97.7
SM (d18:1/C18:0) $m/z = 775$	20.0	18.70 ± 0.57	−6.5	3.1	94.8
	50.0	51.87 ± 1.82	3.7	3.5	91.4
Lyso-PC (C16:0) $m/z = 540$	30.0	28.92 ± 0.63	−3.6	2.2	83.8
	80.0	80.86 ± 4.32	1.1	5.3	94.8

different classes. The different molecular species can be determined by MS.

The molecular mass peaks from the different phospholipid classes were detected using negative-ion full-scan ESI-MS analysis. Negative-ion mode ESI-MS was chosen because it gave more information-rich data with lower noise and background than positive-ion ESI. The species of PG, PE, pPE, PI, and PS were all detected as the $[M-H]^-$ ions. The other three classes (PC, Lyso-PC, and SM) all have a choline group at the polar head and these species were detected as the formate adduct $[M+45]^-$. The molecular mass peaks obtained from these analyses are shown in Fig. 2.

In order to perform species determination of each phospholipid class, we use ion-trap MS^n (MS^2 and MS^3) to detect the fragments in negative-ion mode. The main fragments were lyso-phospholipids and carboxylate anions. Both types of fragments might be used for species determination.

We took PC species for example. In negative-ion ESI/MS mode, PC species were detected as the formate adduct $[M+45]^-$. In the MS spectrum m/z 828.6 $[M+45]^-$ was detected. In the MS^2 spectrum m/z 768.5 $[M-15]^-$ was detected. In MS^3 many lyso-phospholipids and carboxylate anions were detected. Fig. 3 shows the MS^3 spectrum of m/z 828.6 $[M+45]^-$. In the MS^3 spectrum we got the fragment ion at m/z 305.2 (C20:3), 281.4 (C18:1), 279.3 (C18:2), and 255.2 (C16:0), which are the fatty acid fragments. The deacylated ions at m/z 480.4 and 530.9 correspond to Lyso-PC (C16:0) $[M-15]^-$, and Lyso-PC (C20:3) $[M-15]^-$ resulting from the loss of one fatty acid. In Fig. 3 the peak to the left of 305.2 is 303.3 that could be 20:4, but that cannot matches with the other fragments of parent ion 828.6. This phenomenon sometimes happens in the ion-trap MS in which the isolation width is 4m/z, so the 303.3 may be the fragment of 826.5 that was included when the parent ion 828.6 was isolated. The identification of species was based on the molecular ion and *sn*-1 and *sn*-2 carboxylate anions observed. For diacyl species, both carboxylate anions corresponding to *sn*-1 and *sn*-2 substituents were present in the negative-ion mode. The phospholipids isolated from animals often contain a saturated fatty acid in the *sn*-1 position and an unsaturated fatty acid in the *sn*-2 position [26]. However, phospholipids with two unsaturated fatty acids are present in blood, although at a very low concentration with exception of some phosphatidylcholine species [27,28]. According to this rule, the structure for the PC m/z 828.6 may be C18:1/C18:2 or C16:0/C20:3.

More than 100 phospholipids species were identified with the same method described above and results in the published paper. The molecular species with high intensity for the main mass peaks are shown in Table 1.

Table 3
Inter-day accuracy and precision values from the assay QC standards ($n = 6$)

PL class	Low/high concentration (μg/ml)	Measured concentration mean ± RD (μg/ml)	RE (%)	RSD (%)
PE (C18:0/C20:4) $m/z = 766$	10.0	9.67 ± 0.85	−3.3	8.8
	30.0	31.94 ± 1.87	6.5	5.9
PG (C16:0/C18:1) $m/z = 747$	1.0	1.09 ± 0.06	8.8	5.5
	3.0	3.18 ± 0.14	6.1	4.3
PS (C18:0/C18:1) $m/z = 788$	0.5	0.53 ± 0.04	5.4	7.8
	1.5	1.57 ± 0.08	4.4	5.1
PI (C18:0/C20:4) $m/z = 885$	20.0	17.90 ± 1.18	−10.5	6.6
	50.0	46.77 ± 1.47	−6.5	3.1
PC (C18:0/C18:0) $m/z = 834$	2.0	1.88 ± 0.09	−6.0	4.9
	10.0	8.92 ± 0.46	−10.1	5.1
SM (d18:1/C18:0) $m/z = 775$	20.0	18.75 ± 1.45	−6.3	7.7
	50.0	52.35 ± 4.28	4.7	8.2
Lyso-PC (C16:0) $m/z = 540$	30.0	31.18 ± 2.37	3.9	7.6
	80.0	84.83 ± 5.40	6.0	6.4

Table 4
The concentration of different phospholipids species for normal people and DN patients ($p = 0.95$)

PL	Compound	Mean ± s*t/n1/2 normal ($n = 30$)	Mean ± s*t/n1/2 DN-III ($n = 18$)	Mean ± s*t/n1/2 DN-IV ($n = 14$)	Mean ± s*t/n1/2 DN-V ($n = 29$)
PE	$m/z = 766$ (C18:0/C20:4 or C16:0/C22:4)	22.06 ± 2.04	19.72 ± 2.79	23.77 ± 2.26	22.45 ± 2.07
	$m/z = 742$ (C18:0/C18:2 or C18:1/C18:1)	15.69 ± 0.99	15.96 ± 1.62	16.82 ± 2.19	21.64 ± 1.77
	$m/z = 750$ (pC18:0/C20:4 or pC16:0/C22:4)	76.10 ± 5.82	57.87 ± 8.58	37.34 ± 6.77	44.95 ± 4.13
PG	$m/z = 747$ (C16:0/C18:1 or C18:0/C16:1)	1.34 ± 0.10	0.94 ± 0.15	0.75 ± 0.13	0.65 ± 0.07
	$m/z = 745$ (C16:0/C18:2)	0.48 ± 0.04	0.42 ± 0.06	0.37 ± 0.05	0.50 ± 0.05
PS	$m/z = 788$ (C18:0/C18:1 or C16:0/C20:1)	1.41 ± 0.06	1.24 ± 0.09	1.16 ± 0.11	1.21 ± 0.06
	$m/z = 810$ (C18:0/C20:4 or C18:1/C20:3)	2.56 ± 0.09	2.49 ± 0.17	2.38 ± 0.18	2.39 ± 0.13
	$m/z = 834$ (C18:0/C22:6 or C18:1/C22:5)	1.46 ± 0.13	1.46 ± 0.10	1.50 ± 0.18	1.19 ± 0.09
PI	$m/z = 885$ (C18:0/C20:4 or C18:1/C20:3)	38.16 ± 2.83	41.06 ± 2.63	38.84 ± 3.41	28.99 ± 1.94
	$m/z = 861$ (C18:0/C18:2 or C18:1/C18:1)	17.93 ± 0.87	17.48 ± 0.64	18.38 ± 1.48	15.02 ± 0.54
	$m/z = 833$ (C16:0/C18:2 or C16:1/C18:1)	10.88 ± 0.31	11.02 ± 0.22	11.03 ± 0.35	10.36 ± 0.18
PC	$m/z = 834$ (C18:0/C18:0)	11.08 ± 0.39	9.45 ± 0.52	9.48 ± 0.57	7.99 ± 0.52
	$m/z = 802$ (C16:0/C18:2)	634.68 ± 16.95	587.24 ± 29.45	531.1 ± 53.66	505.21 ± 22.01
	$m/z = 854$ (C16:0/C22:4)	131.74 ± 5.22	112.91 ± 6.51	108.57 ± 9.60	105.17 ± 6.35
SM	$m/z = 775$ (dC18:1/C18:0)	30.12 ± 1.15	35.15 ± 3.29	34.53 ± 2.68	27.00 ± 1.47
	$m/z = 747$ (dC18:1/C16:0)	209.70 ± 6.13	230.00 ± 20.08	234.5 ± 21.53	212.5 ± 11.13
	$m/z = 845$ (dC18:1/C23:0)	54.31 ± 1.98	53.45 ± 4.40	61.23 ± 5.61	48.86 ± 2.26
Lyso-PC	$m/z = 540$ (C16:0)	31.58 ± 1.33	34.67 ± 3.56	38.40 ± 4.28	26.46 ± 2.44
	$m/z = 568$ (C18:0)	12.99 ± 0.52	13.29 ± 1.36	14.20 ± 1.24	10.77 ± 1.37
	$m/z = 588$ (C20:4)	6.99 ± 0.26	7.29 ± 0.56	8.00 ± 0.59	6.32 ± 0.42

3.2. Quantification of phospholipids species in seven major phospholipid classes

One representative phospholipids molecule with proper concentration in the plasma in each class were selected for quantification using external calibration method. They are PE (C18:0/C20:4), PG (C16:0/C18:1), PS (C18:0/C18:1), PI (C18:0/C20:4), PC (C18:0/C18:0), SM (d18:1/C18:0), and Lyso-PC (C16:0).

The linear regression analysis was constructed by plotting the peak-area of analytes versus concentration (μg/ml) of analytes. The regression equation of these curves and their correlation coefficients (R) were calculated as follows: PE (C18:0/C20:4), $y = 140773x + 4159.7$, $R^2 = 0.9989$; PG (C16:0/C18:1), $y = 678781x + 46367$, $R^2 = 0.9990$, PS (C18:0/C18:1), $y = 396959x - 6676.4$, $R^2 = 0.9993$; PI (C18:0/C20:4), $y = 377275x - 3082000$, $R^2 = 0.9958$; PC (C18:0/C18:0), $y = 75586x + 10567$, $R^2 = 0.9987$; SM (d18:1/C18:0), $y = 74789x + 13369$, $R^2 = 0.9951$; Lyso-PC (C16:0), $y = 452243x - 586838$, $R^2 = 0.9990$.

The extraction recoveries were determined at two concentration levels and the results are shown in Table 2. For these seven phospholipids the background response in blank plasma was subtracted.

The data from QC samples were calculated to estimate accuracy and intra-day and inter-day precision of the method. The results are presented in Tables 2 and 3.

3.3. Application in DN

The method was applied to diabetic nephropathy. Many studies have demonstrated that diabetic nephropathy is intimately associated with metabolism disorder of lipid or fatty acid in phospholipids. In this paper we want to find out the relationship between diabetic nephropathy and metabolism disorder of phospholipids.

In Section 3.2 we described the method to qualify one representative phospholipids molecule in each class accurately by using standards. Furthermore, other molecular species within the same phospholipid class can also be estimated according to the calibration curves of representative phospholipid since the phospholipids species within the same phospholipid class have the similar structure and similar signal response. And these data is just used for comparing the differences between cases and controls, so the relative quantification data satisfied the estimation. The result is shown in Table 4. Several phospholipids species with high concentration within each class were selected to be shown in the table. Diabetic nephropathy has five development phases in diagnosis (Mogensen phase), in this paper we got the samples on the last three phases (DN-III, DN-IV, and DN-V).

Concentrations of phospholipids species between the controls and diabetic nephropathy patients were various. For single phospholipids species listed in Table 4, basic trend can be seen that the phospholipids within the same class have the similar change trend such as PS, PI, PC, SM, and Lyso-PC. Some single phospholipids species showed obvious change trend which are shown in Fig. 4. With the development of the disease, the concentration of PE $m/z = 750$, PG $m/z = 747$, PC $m/z = 802$ decreased, but the concentration of Lyso-PC $m/z = 540$ increased and then decreased from the controls to DN-III, DN-IV, and DN-V patients.

Adding several phospholipids species in the same class shown in Table 4, the separate concentrations of seven phospholipid classes were got in Fig. 5. For PE, PG, PS, and PC, the concentrations of phospholipids for DN patients are lower than that for controls, especially the concentration of phospholipids for DN-V patient is lowest in almost all the class. For Lyso-PC the concentration increased and then decreased from the controls to DN-III, DN-IV, and DN-V patients. The concentration of SM did not show obvious trend.

Adding all the seven phospholipid classes in Table 4, the total concentration of phospholipids was obtained in Fig. 6. With the development of disease the total concentrations of phospholipids decreased.

For explaining the experiment result, mechanism of DN needs to be reviewed. Many research showed that several pathways have been identified which are activated upon high ambient glucose concentration. These include: activation of the sorbitol pathway, increase in oxidative stress, activation of protein kinase C (PKC), and

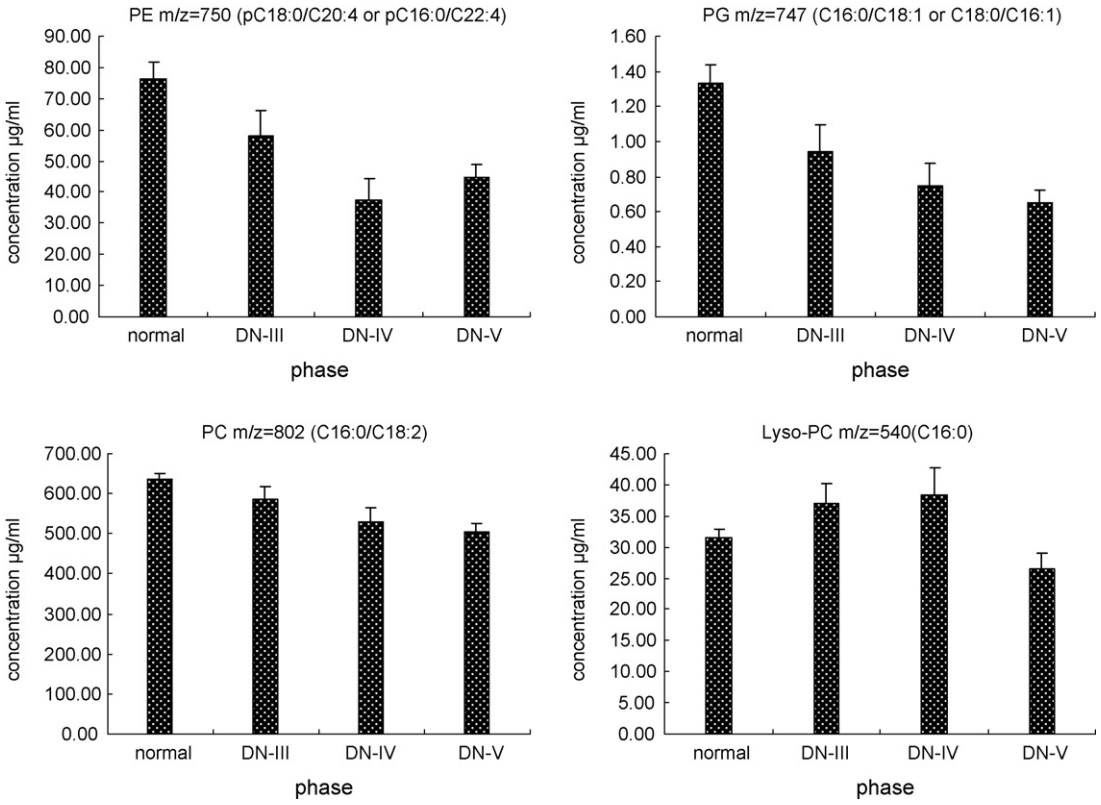

Fig. 4. The comparison of concentration for phospholipids species (PE m/z = 750 pC18:0/C20:4 or pC16:0/C22:4, PG m/z = 747 C16:0/C18:1 or C18:0/C16:1, PC m/z = 802 C16:0/C18:2, Lyso-PC m/z = 540 C16:0) between normal person (n = 30) and DN patients in three phases (DN-III (n = 18), DN-IV (n = 14), and DN-V (n = 29)) (based on the data shown in Table 4).

so on [29–31]. And activation the phospholipase A2 (PLA2) is related with the activation of protein kinase C (PKC). Phospholipases are important enzyme in the body that can catalyze the decomposition of phospholipids to produce free fatty acids. So the activated phospholipase A2 will accelerate the decomposition of phospholipids. The result of our experiment is consistent with this mechanism. With the development of DN the PLA2 was activated so the concentrations of phospholipids decreased. For Lyso-PC which was produced by PC losing one fatty acid chain under the catalysis of phospholipase, so when PC decomposed, the concentration for Lyso-PC increased in DN-III and DN-IV. Then Lyso-PC decreased in DN-V because of decomposition and low concentration of PC class. SM has different metabolism path and it has little relationship with this mechanism, so the concentration of SM did not show obvious trend.

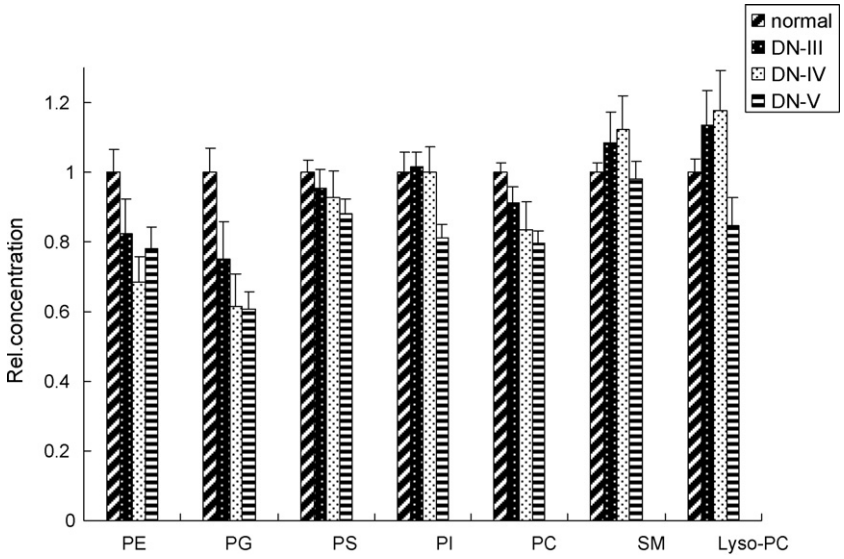

Fig. 5. The comparison of relative concentration (set the concentration of normal at 1) for phospholipid classes (PE, PG, PS, PI, PC, SM, and Lyso-PC) between normal person (n = 30) and DN patients in three phases (DN-III (n = 18), DN-IV (n = 14), and DN-V (n = 29)) (adding the concentration of several phospholipids species in the same class shown in Table 4 to get the concentration of phospholipid class).

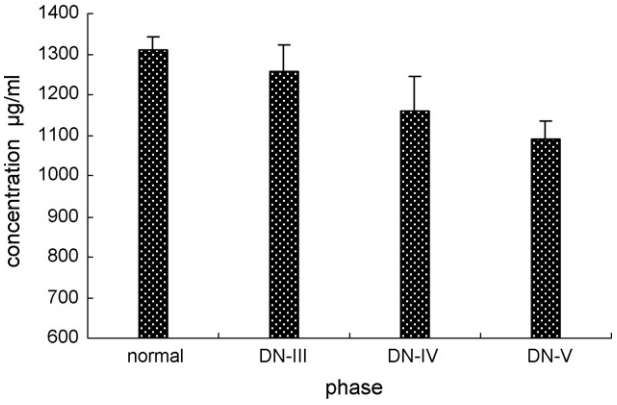

Fig. 6. The comparison of total concentration of phospholipids between normal person ($n = 30$) and DN patients in three phases (DN-III ($n = 18$), DN-IV ($n = 14$), DN-V ($n = 29$)) (adding the concentration of all the seven phospholipid classes in Table 4 to get total concentration of phospholipids).

4. Conclusion

In conclusion, this paper described a rapid, and specific analytical method for simultaneous determination and estimation of seven major phospholipid classes in human blood by normal-phase high-performance liquid chromatography–tandem mass spectrometry. Isocratic elution method was used for better repeatability and no balance time which has advantages for high-through analysis. Through the accurate quantification of one representative phospholipids molecule in each class a method for simultaneous estimation hundreds of molecular species in seven major classes was established. The method is efficient and economical which can be used in qualifying other compound classes which have similar structure. Though only the relative quantification data can be obtained, it satisfied the estimation. The method established has been successfully applied to the research in diabetic nephropathy.

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