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Analysis of phospholipid species in human blood using normal-phase liquid chromatography coupled with electrospray ionization ion-trap tandem mass spectrometry

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Abstract

A narrow-bore normal-phase high-performance liquid chromatography (HPLC) method was developed for separation of phospholipid classes in human blood. The separation was obtained using an HPLC diol column and a gradient of chloroform and methanol with 0.1% formic acid, titrated to pH 5.3 with ammonia and added 0.05% triethylamine. The HPLC system was coupled on-line with an electrospray ionisation ion-trap mass spectrometer. Chromatographic baseline separation was obtained between phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, lyso-phosphatidylcholine, phosphatidylinositol and phosphatidylserine, eluting in that order. The total run time was 30 min. Plasmalogen phosphatidylethanolamine and sphingomyelin, which both are substances with structural similarities to the glycerophospholipids, had similar retention time as phosphatidylethanolamine, but were well separated from the other glycerophospholipid classes. The species from each class were identified using MS^2 or MS^3, which forms characteristic lyso-fragments. The combination of lyso-fragment mass, molecular ion and chromatographic retention time was used to identify each species, including 20 species of phosphatidylglycerol. The mass spectra obtained for the phospholipid classes are presented. Using this system 17 disaturated phospholipid species not earlier described to be present in blood were identified. The limit of detection varied between different phospholipid classes and was in the range 0.1–5 ng of injected substance.

Keywords: Phospholipids

1. Introduction

Phospholipids are the main constituents of biological membranes. In addition to having this important role for the structural and functional properties of the cell membrane of all living organisms, the phospholipids also play a main role in signal processing and as a precursor for many other biologically important molecules. The phospholipids are divided into several classes based on differences in the polar head groups. These different classes of phospholipids are unevenly distributed on the two leaflets of cell membranes and also between different organs [1]. Each class of phospholipids is composed of a mixture of many molecular species containing different fatty acids.

During the last two decades there has been an

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increased interest for using phospholipid vesicles, i.e. liposomes, as a drug carrier system. Analyses of the different classes and species of phospholipids in biological material are thus an important issue both for basic research and for the pharmaceutical industry. The increased commercial use of phospholipids as constituents of drugs has resulted into an increased requirement for specific analytical methods to separate and identify the phospholipids added to drugs from the endogenous phospholipids in blood in order to perform pharmacokinetic and toxicokinetic analyses as part of product documentation.

The phospholipids isolated from animals most often contain a saturated fatty acid in the *sn*-1 position and an unsaturated fatty acid in the *sn*-2 position [1]. However, phospholipids with two unsaturated fatty acids are present in blood, although at a very low concentration with exception of some phosphatidylcholine species [2,3]. The phospholipids used in drugs most often contain two saturated fatty acids as this reduces the risk for oxidation of the product. Only some few species of such disaturated phospholipids have been reported to be present in blood [4,5], whereas disaturated phosphatidylcholine species have for many years been known to be responsible for the reduction of surface tension in alveoli and maintaining alveolar stability in the lung [6].

Despite the importance of good methods for phospholipid analyses, relative little attention has up to now been paid upon analysis of the molecular species of the different phospholipid classes. The reason for this is probably that separation and detection of these compounds have up to recently been relative complicated and time consuming. Thus, the method of choice was for many years to use thin layer chromatography for class separation, followed by extraction and hydrolysis of the phospholipids and the use of gas chromatography [7] or high-performance liquid chromatography (HPLC) [4] for identification of covalently modified derivatives. Normal-phase HPLC has now been used extensively to separate the phospholipids into different classes (for review see Refs. [8,9]), using UV absorbance, fluorescence, refractive index, flame ionisation and evaporative light scattering as detection methods [10]. Reversed-phase HPLC separates the phospholipids into different molecular species [11], but this method often requires the use of ion-pair reagents which may limit the detectors that can be used [12,13].

The use of mass spectrometry (MS) detection is a very useful approach to increase the specificity of the phospholipid analyses, and HPLC–MS is now routinely used for lipid analysis [14]. This approach makes it possible to quantitate specific phospholipid species on-line. However, it is important to have a chromatographic system, which separates the different phospholipid classes due to possible mass overlap. We have earlier described normal-phase HPLC systems for separation of phospholipid classes [15,16]. In the present work we describe an improved chromatographic method for class separation of phospholipids in blood. Coupling this chromatographic method with on-line ion-trap MS detection, made it possible to detect many phospholipid species not earlier described to be present in blood. The method should also be useful for analysing phospholipid species from other sources.

2. Experimental

2.1. Materials

Chloroform stabilised with ethanol, formic acid and triethylamine were HPLC grade from Rathburn (Walkerburn, UK). Methanol was LiChrosolv grade and ammonia (25%) pro-analysis grade from Merck (Darmstadt, Germany); 2,6-di-*tert*-butyl-4-methylphenol was from Aldrich-Chemie (Steinheim, Germany). 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phospho-L-serine (PSPS) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) were from Avanti Polar Lipids (Alabaster, AL, USA). Bovine liver phosphatidylinositol (PI, i.e. a mixture of different species), bovine erythrocytes spingomyelin (SM, i.e. a mixture of different species) and 1-stearoyl-2-lyso-*sn*-glycero-3-phosphocholine (lyso-SPC) were from Sigma Chemical Company (St. Louis, MO, USA). 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoglycerol (DSPG) were from Lipoid GmbH (Ludwigshafen, Germany). Bovine brain plasmalogen phosphatidylethanolamine (pPE, i.e. a mixture of different species) was from Larodan Fine

Chemicals (Malmø, Sweden). According to the manufacturers, the purity of the standard compounds was approximately 99% (pPE was 90%).

2.2. Sample preparation

The standards were dissolved (approx. 1 mg/ml) in chloroform–methanol–water (65:25:4, v/v), and further diluted (0.01–10 μg/ml) in chloroform–methanol (95:5, v/v). The dissolved standards were stored in refrigerator.

Heparinised human whole blood was pooled from three fasting male subjects. The lipids in the samples were extracted essentially as described earlier [16]. Briefly, 0.7 ml of water was added to 300 μl of the blood sample; then 5 ml of methanol with 0.01% (w/v) 2,6-di-*tert*-butyl-4-methylphenol and 10 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, 5 ml of water was added before the solution was mixed for 5 s and centrifuged at 2600 *g* 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 300 μl of chloroform–methanol (95:5, v/v), of which 10 μl was injected into the chromatograph.

2.3. Chromatographic conditions

An HP 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) was used. The lipids were separated on a LiChroCART, LiChrospher 100 Diol, 250×2 mm, (5 μm) column with a LiChrospher 100 Diol, 10×2 mm, (5 μm) precolumn (Merck, Darmstadt, Germany). Chloroform was used as mobile phase A. Mobile phase B was methanol with 0.1% (v/v) formic acid, ammonia added to pH 5.3 (approx. 0.05%, v/v of ammonia) and 0.05% (v/v) triethylamine. Separation was obtained by using a gradient starting at 95% mobile phase A, decreasing to 70% A in 11 min and further decreasing to 20% A in 3 min, held at 20% A in 4 min and then back to 95% A in 2 min. The total chromatographic run time was 30 min. The flow-rate was 0.3 ml/min and the column temperature was 30°C.

2.4. Mass spectrometry

The HPLC system was coupled on-line to an LCQ ion-trap mass spectrometer (Finnigan, San Jose, CA, USA) equipped with an electrospray (ES) ionization source. The HPLC effluent entered the MS through a steel ES ionisation needle set at 4.5 kV and a heated capillary set to 230°C. The sheath gas flow was approx. 90 units. The ion source and ion optic parameters were optimised with respect to the negative molecular related ions of the phospholipid standards. Mass resolution was 0.6 Da at half peak height and isolation width ±0.5 Da. MS^n experiments were carried out with relative collision energy of 30–40% and the trapping of product ions were carried out with a *q* value of 0.25.

3. Results and discussion

3.1. Separation of phospholipid classes

Initially, mobile phase B (see Experimental section) consisted of methanol with 0.2% (v/v) formic acid titrated to pH 5.3 with ammonia. This did not, however, give an optimal separation of the different phosphoplipid classes and also caused problems with precipitation at the ion source heated capillary. A decrease in the amounts of formic acid and ammonia to avoid precipitation and addition of a small amount of triethylamine resulted in improved separation between the five main phospholipid classes of human blood as shown in Fig. 1A. The retention times for the different classes increased in the order phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS). All classes eluted within 20 min and the total run time was 30 min.

PC is the most common phospholipid in blood, resulting in rather high concentrations of lyso-PC. Fig. 1B shows that lyso-PC in this system is separated from PC and elutes between PE and PI. Sphingomyelin (SM) and plasmalogen PE (pPE) are other substances present in blood that show large structural similarities with the diacylphospholipids. The chromatogram in Fig. 1B shows that pPE and SM elutes close to PE. As shown below, the on-line MS detection allows selective mass fragment analy-

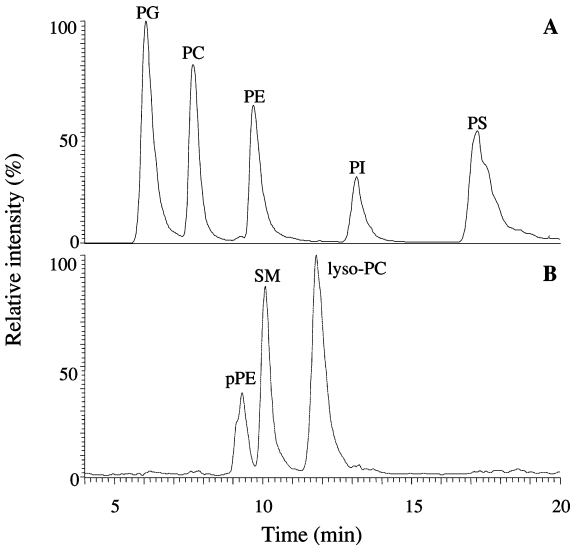

Fig. 1. Negative-ion HPLC-ES-MS analysis of a phospholipid mixture (approx. 10 μg/ml of each) with the MS operating in the scanning mode. (A) The sum of mass chromatograms of DSPG (*m*/*z* 777.6), DSPC (*m*/*z* 834.6), POPE (*m*/*z* 716.5), PI (*m*/*z* 885.5, i.e. C18:0/C20:4 in the extract) and PSPS (*m*/*z* 762.5). (B) The sum of mass chromatograms of pPE (*m*/*z* 750.6, i.e. C18:0/C20:4 and C16:0/C22:4 in the extract), SM (*m*/*z* 747.5, i.e. C16:1/C16:0 (base/amide) in the extract) and lyso-PC (*m*/*z* 508.3, i.e. C18:0). Conditions given in Experimental section.

sis of the different species of PE and pPE and made it possible to identify these species from the SM species.

Although the chromatographic system mainly separates the different phospholipid classes it will also give some minor separation between different species within a given class. Thus, Fig. 2A–C show the chromatograms obtained when using the MS to select the signals from three different species of PC from a full-scan MS analysis of human blood. These three chromatograms illustrate that some differences in retention times are obtained for different species within a given class. However, the difference in retention time between species within a given class is less than the difference in retention time between each class as shown in Fig. 2D. Hence, the chromatographic retention times can be used to identify each class and most importantly the system separates isobaric species from different phospholipid classes.

3.2. Species characterisation of the phospholipid classes

The molecular mass peaks from the different phospholipid classes were detected using negative ion full-scan ES-MS analysis. The species of PG, PE, pPE, PI and PS were all detected as the $[M–H]^-$ ions. The three other classes (PC, lyso-PC and SM) all have a choline group at the polar head and these species were best detected as the formate adduct, i.e. $[M+45]^-$. It should be noted that minor peaks of these three classes were obtained at $[M+35]^-$ (chlorine adduct) and $[M–15]^-$ (due to loss of CH_3 from the choline group). The molecular mass peaks obtained from these analyses are shown in Fig. 3.

In order to perform species determination of each phospholipid class, negative ion fragments (MS^2 and MS^3) formed after collision-induced dissociation was detected. The main fragments were lyso-phospholipids and carboxylate anions. Both type of fragments might be used for species determination. However, with the ion-trap MS the lyso-phospholipid fragments were the most abundant signals and were used for species determination (Table 1).

The ion-trap MS was set in the full-scan MS^3 mode for PS and PC and full-scan MS^2 mode for the other classes. It should be noted that it is possible to perform such analysis in the MS^2 mode also for PS and PC by using source fragmentation, but the use of MS^3 mode for PS and PC was chosen due to a better selectivity. It should also be noted that PE and pPE gave different product ion scan mass spectra and thus could be easily distinguished. The detailed interpretation of the fragments formed with the different phospholipids using the ion-trap MS and comparison of these fragments with those obtained using a triple quadrupole MS will be published elsewhere.

The molecular species identified as responsible for the main mass peaks and their relative intensities of these mass peaks are shown in Table 2; included in Table 2 are also the results from species characterisation of disaturated phospholipids present in rather low amounts in blood (see below). It should be noted that species characterisation was not performed for SM (the peaks in Fig. 3C with *m*/*z* values above 800; the SM species have *m*/*z* values where the first digit before the decimal point is an odd number), as

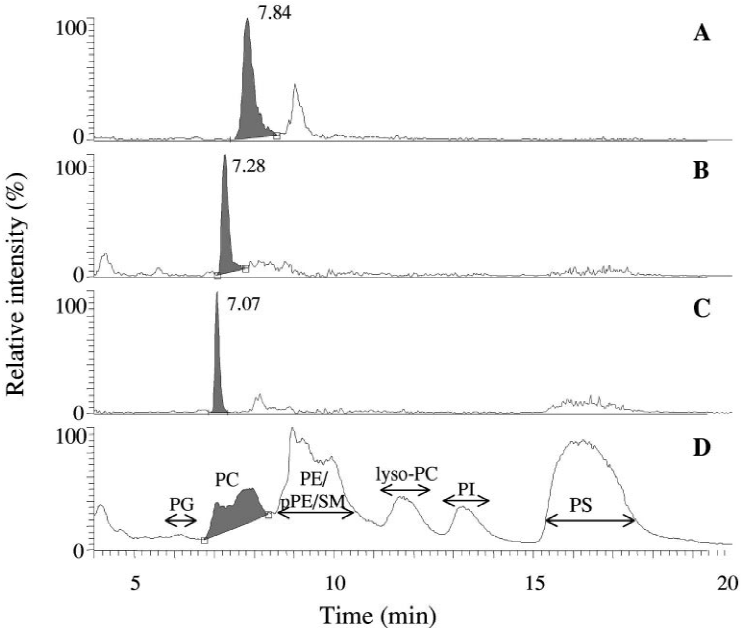

Fig. 2. Negative-ion HPLC-ES-MS analysis of extracted human blood with the MS operating in the scanning mode. (A–C) Mass chromatograms of three PC species; (A) m/z 778.5, i.e. C16:0/C16:0; (B) m/z 826.5, i.e. C16:0/C20:4; (C) m/z 878.5, i.e. C18:0/C22:6. (D) Total ion current from m/z 450 to 950; the shadowed peaks are representing the molecular species of PC; the bars indicate the elution times of the other classes. Conditions given in the Experimental section.

electrospray ionisation does not give negative ion fragments. However, SM species may be characterised using atmospheric pressure chemical ionisation [17].

The relative peak areas in Table 2 give an indication about the relative abundance of the different species. However, the distribution between the isobaric species is not given. It should be noted that the MS signal intensities varies between different classes and between different species within a given class such that standards need to be run for each species to obtain absolute quantification. According to Schwarz et al. [18], the concentrations of the phospholipid classes in human blood are 1450 μM PC, 473 μM SM, 175 μM PE, 114 μM PI, 90 μM PS and 63 μM PG (approx. 2900 μM phospholipids in blood; all data recalculated from plasma values and blood cell values based on a blood cell volume of 46%).

The very good chromatographic separation obtained with the present system made it possible to change the MS settings as a function of retention time for each of the five main phospholipid classes in one single run. This possibility is illustrated in Fig. 4, which shows product ion spectra obtained from different time windows (to obtain as many data points as possible for each peak) within a single run of extracted human blood (MS^3 for PS and PC; MS^2 for the others). The examples shown in Fig. 4 are all disaturated phospholipids, several of them present in very low amounts as shown in Table 2. It should be noted that the spectra for PC in Fig. 4B (i.e. at m/z 722.5) show the presence of both C14:0/C14:0 and C12:0/C16:0, which have the same molecular mass. Similarly, both the C16:0/C16:0 and C14:0/C18:0 species were observed for PI as shown in Fig. 4D.

The product ion spectra in Fig. 4 illustrate that it is possible to detect and quantitate phospholipid species from each class of PG, PC, PE, PI and PS in one single run. Based on the separation shown in Fig. 1, it is possible to include species of lyso-PC in such an analysis. It should also be possible to analyse even

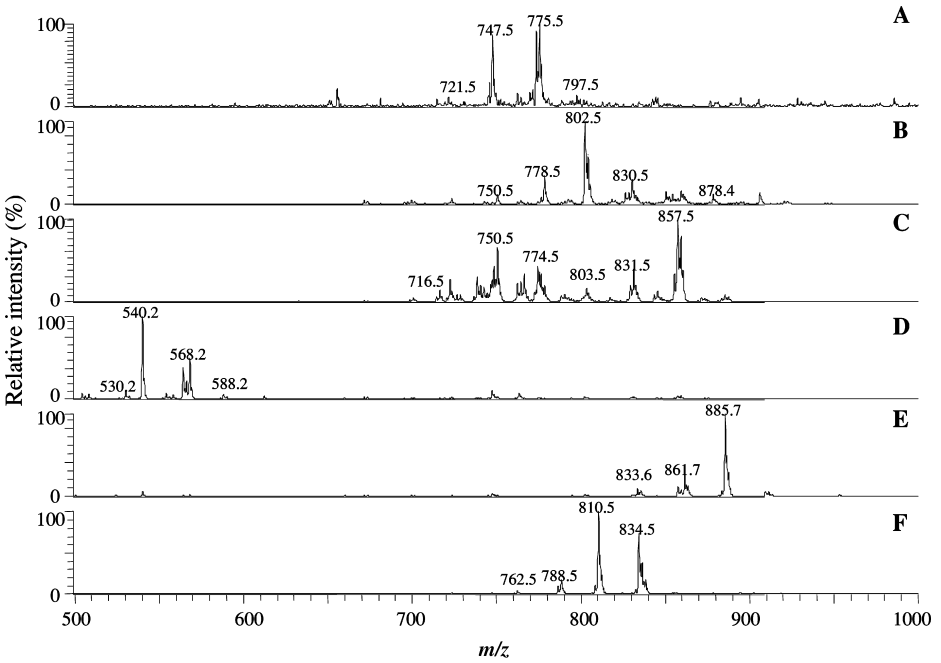

Fig. 3. Negative-ion HPLC-ES-MS analysis of extracted human blood. Mass spectrum of the substance classes shown in Fig. 2D: (A) PG; (B) PC; (C) PE/pPE/SM; (D) lyso-PC; (E) PI; (F) PS.

more species in one single run, although this may result in a decreased sensitivity.

3.3. Species characterisation of disaturated phospholipids

The spectra shown in Fig. 4 indicate the presence of disaturated phospholipids in all phospholipid classes. The identification of disaturated phospholipids may be complicated, however, due to effect of naturally occurring isotopes (mainly the presence of 1.1% of ^{13}C). This isotope effect cause problems for detection of disaturated phospholipids containing C18:0 due to the presence of large amounts of phospholipids containing C18:1. Thus, by setting the peak area of the m/z ion of a phospholipid containing C18:0/C18:1 to 100%, the isotope effect will result in a peak of approx. 44% at one mass unit

Table 1
Molecular ions and fragment ions of phospholipids used in species characterisation[a]

PL class	Molecular ions	Main fragments detected by MS^2	Main fragments detected by MS^3
PG	$[M-H]^-$	$[lyso\text{-}PG-H]^-$, $[lyso\text{-}PG-H-H_2O]^-$, $[lyso\text{-}PA-H-H_2O]^-$*, $[FA-H]^-$	
PC	$[M+45]^-$	$[M-CH_3]^-$	$[lyso\text{-}PC-CH_3]^-$*, $[FA-H]^-$
PE	$[M-H]^-$	$[lyso\text{-}PE-H]^-$*, $[FA-H]^-$	
pPE	$[M-H]^-$	$[lyso\text{-}pPE-H]^-$*, $[FA-H]^-$	
lyso-PC	$[M+45]^-$	$[M-CH_3]^-$	$[FA-H]^-$*
PI	$[M-H]^-$	$[lyso\text{-}PI-H-H_2O]^-$, $[lyso\text{-}PA-H-H_2O]^-$*	
PS	$[M-H]^-$	$[PA-H]^-$	$[lyso\text{-}PA-H-H_2O]^-$*

[a] MS^2 was used for PG, PE, pPE and PI; MS^3 was used for PC, lyso-PC and PS. * Indicate which fragments that were used for species identification. PL, phospholipid; PA, phosphatidic acid; FA, fatty acid.

Table 2
Identification of the main phospholipid species and detected disaturated phospholipids in a pooled human blood sample

Phospholipid class	Ions (*m*/*z*)	Relative intensities (%)	Combinations of molecular species		
PG	821.5	4	C18:0/C22:6		
	801.5	9	C18:0/C20:2	C18:1/C20:1	C18:2/C20:0
	797.5	15	C18:0/C20:4		
	777.5	13	C18:0/C18:0 (1.3)		
	775.5	100	C18:0/C18:1	C16:0/C20:1	
	773.5	85	C18:0/C18:2	C18:1/C18:1	C16:0/C20:2
	769.5	20	C16:0/C20:4		
	749.5	20	C16:0/C18:0 (2.3)		
	747.5	90	C16:0/C18:1	C18:0/C16:1	
	745.5	30	C16:0/C18:2		
	721.5	12	C16:0/C16:0		
	719.5	6	C16:0/C16:1		
	693.5	1	C14:0/C16:0*		
	665.5	<1	C14:0/C14:0*		
PC	878.5	10	C18:0/C22:6		
	854.5	10	C18:0/C20:4	C16:0/C22:4	
	850.5	15	C16:0/C22:6		
	830.5	30	C18:0/C18:2	C18:1/C18:1	C16:0/C20:2
	828.5	15	C16:0/C20:3	C18:1/C18:2	
	826.5	10	C16:0/C20:4	C18:2/C18:2	
	806.5	10	C16:0/C18:0 (1.6)		
	804.5	60	C16:0/C18:1		
	802.5	100	C16:0/C18:2		
	792.5	6	C16:0/C17:0		
	778.5	35	C16:0/C16:0		
	776.5	8	C16:0/C16:1		
	764.5	5	C15:0/C16:0		
	750.5	10	C14:0/C16:0		
	722.5	3	C14:0/C14:0	C12:0/C16:0	
PE and pPE	778.5	35	pC18:0/C22:4	pC20:0/C20:4	
	774.5	65	pC18:0/C22:6	pC18:1/C22:5	
	766.5	55	C18:0/C20:4	C16:0/C22:4	
	764.5	50	C18:1/C20:4	C16:0/C22:5	C18:0/C20:5
	762.5	35	C16:0/C22:6		
	750.5	100	pC18:0/C20:4	pC16:0/C22:4	
	746.5	30	pC16:0/C22:6	pC18:1/C20:5	
	744.5	15	C18:0/C18:1		
	742.5	25	C18:0/C18:2	C18:1/C18:1	C16:0/C20:2
	740.5	30	C18:1/C18:2	C16:0/C20:3	
	738.5	45	C16:0/C20:4	C18:2/C18:2	
	716.5	20	C16:0/C18:1		
	714.5	6	C16:0/C18:2		
	690.5	<1	C16:0/C16:0		
	676.5	<1	C15:0/C16:0*		
	662.5	<1	C14:0/C16:0		
	634.5	<1	C14:0/C14:0*	C12:0/C16:0*	

Table 2. Continued

Phospholipid class	Ions (m/z)	Relative intensities (%)	Combinations of molecular species		
lyso-PC	612.3	3	C22:6		
	590.3	3	C20:3		
	588.3	6	C20:4		
	586.3	1	C20:5		
	582.3	<1	C19:0		
	568.3	50	C18:0		
	566.3	25	C18:1		
	564.3	40	C18:2		
	554.3	1	C17:0		
	540.3	100	C16:0		
	538.3	1	C16:1		
	526.3	1	C15:0		
	512.3	1	C14:0		
PI	913.5	3	C18:0/C22:4	C20:0/C20:4	
	911.5	6	C18:0/C22:5	C18:1/C22:4	C20:1/C20:4
	909.5	7	C18:0/C22:6	C18:1/C22:5	
	887.5	30	C18:0/C20:3		
	885.5	100	C18:0/C20:4	C18:1/C20:3	C16:0/C22:4
	883.5	7	C18:1/C20:4	C16:0/C22:5	C18:0/C20:5
	865.5	1	C18:0/C18:0 (1.3)		
	863.5	10	C18:0/C18:1	C16:0/C20:1	
	861.5	25	C18:0/C18:2	C18:1/C18:1	C16:0/C20:2
	859.5	7	C16:0/C20:3	C18:1/C18:2	C18:0/C18:3
	857.5	11	C16:0/C20:4	C18:2/C18:2	
	837.5	1	C16:0/C18:0 (1.9)		
	835.5	7	C16:0/C18:1	C16:1/C18:0	
	833.5	8	C16:0/C18:2	C16:1/C18:1	
	823.5	<1	C16:0/C17:0*	C15:0/C18:0*	
	809.5	<1	C16:0/C16:0	C14:0/C18:0	
	781.5	<1	C14:0/C16:0*		
PS	838.5	15	C18:0/C22:4	C20:0/C20:4	
	836.5	35	C18:0/C22:5	C18:1/C22:4	C20:1/C20:4
	834.5	75	C18:0/C22:6	C18:1/C22:5	C20:2/C20:4
	832.5	5	C18:1/C22:6	C20:3/C20:4	
	818.5	<1	C18:0/C20:0		
	810.5	100	C18:0/C20:4	C18:1/C20:3	C16:0/C22:4
	808.5	10	C18:1/C20:4	C18:0/C20:5	C16:0/C22:5
	790.5	2	C18:0/C18:0 (1.3)		
	788.5	20	C18:0/C18:1	C16:0/C20:1	
	786.5	10	C18:0/C18:2	C18:1/C18:1	
	784.5	1	C18:1/C18:2	C16:0/C20:3	C18:0/C18:3
	782.5	1	C16:0/C20:4		
	762.5	4	C16:0/C18:0		
	760.5	<1	C16:0/C18:1	C16:1/C18:0	
	748.5	<1	C16:0/C17:0	C15:0/C18:0	
	734.5	<1	C16:0/C16:0	C14:0/C18:0	

The identification was based on a combination of retention time, molecular ion and product ion scan of the main signals obtained in full scan mode of each class. The relative intensities of the molecular ion peaks within each phospholipid class are also given, such that the main peak within each class is set to 100; the relative intensities are not corrected for the ^{13}C effect. The number in parenthesis show the ratio of the measured peak height to that obtained from the isotope effect of the corresponding monounsaturated species for those disaturated phospholipids where the peaks were only somewhat (between 1.3 and 3 times) larger than that due to the isotope effect. The * is used to mark those disaturated phospholipids which have peaks too low to calculate the ratio compared to that of the isotope effect.

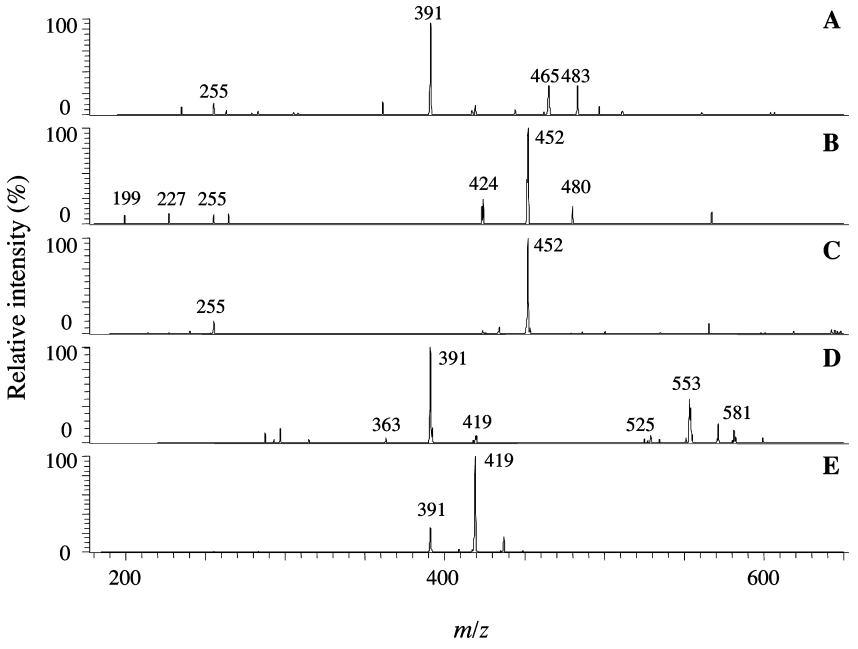

Fig. 4. Negative-ion HPLC-ES-MS^n analysis of extracted human blood. The product ion spectra at one m/z value from each of the glycerophospholipid classes were obtained in one single run. (A) MS^2 of PG (from 0 to 6.7 min; m/z 721.5) showing the C16:0/C16:0 fragments; (B) MS^3 of PC (from 6.7 to 8.7 min; m/z 722.5) showing the C14:0/C14:0 and C12:0/C16:0 fragments; (C) MS^2 of PE (from 8.7 to 11.7 min; m/z 690.5) showing the C16:0/C16:0 fragments; (D) MS^2 of PI (from 11.7 to 14.0 min; m/z 809.5) showing the C16:0/C16:0 and C14:0/C18:0 fragments; (E) MS^3 of PS (from 14.0 to 22.0 min; m/z 762.5) showing the C16:0/C18:0 fragments. Identification of species was obtained using the lyso-fragments listed in Table 1.

above and a peak of approx. 12% at two mass units above (these numbers differ somewhat dependent upon the phospholipid class and bound fatty acids). This means that the peak at the mass of e.g. C18:0/C18:0 within a given phospholipid class needs to be larger than 12% of the peak at the mass of C18:0/C18:1 for the same phospholipid class to show the presence of the disaturated phospholipid.

In order to detect disaturated phospholipids using the present HPLC–MS system the theoretical mass of all disaturated phospholipid combinations from C12:0/C12:0 up to C20:0/C22:0 (increase of two carbon atoms between each analysis performed for PG, PC, PE, PI and PS) were looked for using product ion scan. If the product ion scan indicated the presence of a given disaturated species, the full scan signal at the theoretical mass was compared with the signals at one and two masses below. In addition to performing these analyses with the even number fatty acids mentioned above, we included two combinations containing one odd number fatty acid, i.e. C15:0/C16:0 and C16:0/C17:0 as Myher et al. [4] have described very low amounts of these two PC species to be present in human erythrocytes.

For PG the peak at m/z 721.5 was higher than the peak at m/z 720.5 showing the true presence of C16:0/C16:0. Similar results were obtained with C14:0/C14:0 and C14:0/C16:0. When doing similar analysis for the PG peaks at m/z 749.5 (C16:0/C18:0) and m/z 777.5 (C18:0/C18:0) these peaks were 27 and 17%, respectively, of that obtained when measuring at two mass units below. This correspond to 2.3 and 1.3 times the peaks expected purely based on the isotope effects of the corresponding phospholipid where one C18:0 has been exchanged with C18:1. Thus these analyses indicate the presence of these disaturated PG species, but at the same time illustrate the problem of analysing disaturated phospholipids containing C18:0 with this technique due to the large amounts of C18:1. To our knowledge this is the first demonstration of disaturated PG species in blood.

Similar analyses to that described for PG was performed for all other phospholipid classes. For PC, there were no doubt about the presence of C12:0/C16:0 and C14:0/C14:0, which have not been described earlier to be present in blood. Furthermore, C14:0/C16:0, C15:0/C16:0 and C16:0/C16:0 were clearly identified in accordance with that reported by Myher et al. [4]. The PC peak at m/z 806.6 (C16:0/C18:0) was, however, only 1.6 times that of the isotope effect from C16:0/C18:1. This once again illustrates the problems with detection of disaturated phospholipids containing C18:0, as Myher et al. [4,5] have reported the presence of both C16:0/C18:0 and C18:0/C18:0 PC species in blood.

For PE, five disaturated species were identified: C12:0/C16:0, C14:0/C14:0, C14:0/C16:0, C15:0/C16:0 and C16:0/C16:0. The four first mentioned have not earlier been described to be present in blood. Myher et al. [4,5] reported the presence of three disaturated PE species, i.e. C16:0/C16:0, C16:0/C18:0 and C18:0/C18:0.

For PI the following disaturated species were identified:C14:0/C16:0, C14:0/C18:0, C16:0/C16:0, C16:0/C17:0 and C15:0/C18:0, of which only C16:0/C16:0 has earlier been described to be present in blood [4]. The PI peaks at m/z 837.5 (C16:0/C18:0) and m/z 865.6 (C18:0/C18:0) were 1.9 and 1.3 times, respectively, of that expected purely from the isotope effects of the peaks obtained at two masses below. These two species have been described to be present in blood in very low amounts [4,5].

For PS the following species were identified: C14:0/C18:0, C16:0/C16:0, C16:0/C17:0, C15:0/C18:0, C16:0/C18:0 and C18:0/C20:0, whereas the peak with m/z 790.5 (C18:0/C18:0) was 1.3 times that expected purely from the isotope effect of the peak obtained at two masses below. Only two of these PS species have earlier been described to be present in blood, as Myher et al. [4] reported low amounts of C16:0/C16:0 and C16:0/C18:0 in their very detailed characterisation of human erythrocyte and plasma phospholipids. It should be noted that in order to perform that detailed characterisation they used the very time consuming method of thin layer chromatography to obtain class separation, followed by extraction and treatment with phospholipase C and finally identification of covalently modified derivatives by gas chromatography.

3.4. Sensitivity

The sensitivity of the present method is mainly dependent upon which class of phospholipids that is analysed, although there is also some variation between the different species within a given phospholipid class. By making dilutions of the solutions used to obtain the chromatogram shown in Fig. 1A, the following LOD values (signal to noise better than 3) were estimated (values given as amount of injected substance): 0.1 ng DSPG, 0.5 ng DSPC, 0.5 ng POPE, 5 ng PI (estimated from the peak of C18:0/C20:4) and 5 ng PSPS. Similar values were obtained whether these analyses were performed in the full-scan or SIM (single ion monitoring) modes.

3.5. Overall discussion and conclusions

The HPLC–MS method described in this paper gives an excellent separation of the different phospholipid classes, and was used to identify many phospholipid species not earlier described to be present in human blood. In the present study, we selected to use an extraction procedure for human blood similar to one we have used earlier [16]. It should be noted that Eder et al. [19] have published a very detailed work on the use of different solutions and extraction times to obtain the best possible recovery depending upon which class of phospholipid the analyses is mainly focusing upon.

Using the present method the intensity of the MS signals varies between different phospholipid species. Thus, the LOD values estimated for one species from each of the main classes differed by a factor of 50 with LOD values in the range 0.1–5 ng. If the goal is to measure one single species only, it is likely that the sensitivity can be improved somewhat by optimising the system for that compound. It should be noted that it is necessary to use standards for each species to obtain correct quantification. Based on this background and since as many as 151 species are listed in Table 2, it was not our aim to focus on absolute quantification of the different species. Thus, in contrast to our earlier work showing complete validation results for one PS species using a single quadrupole LC–MS system [16], the intention with the present work was to demonstrate the potential of using the ion-trap for detection and identification of phospholipid species in blood.

Using the present HPLC–MS system we were able to detect 25 disaturated phospholipid species, of which 17 have not earlier been described to be present in blood. Another six disaturated phospholipid species, four of these earlier reported to be present in blood [4,5], were detected in amounts corresponding to only 1.3–2.3 times the peak expected purely from the isotope effect of the peaks obtained at two masses below. In Table 2 there is a total of 151 species identified, although it was not our intention to make this table complete, i.e. the species listed in this table are either from the main peaks within a given class or those obtained when looking specifically for disaturated phospholipids. To our knowledge characterisation of PG species in blood have not been described earlier, whereas we in the present work identified 20 such species. The lack of earlier data on PG species in blood may partly be due to the low concentration of PG in blood; according to Schwarz et al. [18] PG constitute approx. 2% of the total phospholipids in blood. The lack of such data may, however, also be due to that full-scan screening are less sensitive than SIM detection when using quadrupole MS (our unpublished data), i.e. the MS system most commonly used up to now. On the other hand, the sensitivity of analysing phospholipids using the LCQ detector is very similar whether the analyses are performed in the full-scan MS, full-scan MS^n or SIM modes.

The present HPLC–MS system has shown to be very convenient when analysing disaturated phospholipids with fatty acids not longer than C17:0. The isotope effect on the mass spectra, mainly due to ^{13}C, together with the large amounts of C18:1 makes it difficult, however, to identify low amounts of disaturated phospholipids containing C18:0. This problem was, however, less for PS and PI than for the other phospholipid classes. An obvious way to be able to detect even more phospholipid species is to use the present chromatographic system to fractionate the different phospholipid classes, and then use a reverse phase HPLC–MS system to separate the different species within each phospholipid class. Such a two dimensional chromatographic system would, however, be much more time consuming and thus less useful especially for analysis of the large number of samples normally obtained from pharmacokinetic and toxicokinetic studies during drug development.

Finally, it should be mentioned that triethylamine have been reported to adsorb to the surface of the vacuum manifold of an ion-trap MS [20] similar to that used in the present study. This adsorption was reported to result into problems when using the instrument in the positive ion mode for low molecular mass substances due to the triethylamine signal at m/z 102. We have, however, not observed any major problems with our instrument after running the present method for many hundred of samples.

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