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Determination of nonpolar and polar lipid classes in human plasma, erythrocytes and plasma lipoprotein fractions using ultrahigh-performance liquid chromatography-mass spectrometry

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ABSTRACT

A novel normal-phase (NP) ultrahigh-performance liquid chromatography-mass spectrometry (UHPLC/MS) method is developed for a separation and quantitation of nonpolar lipid classes occurring in human plasma, erythrocytes and plasma lipoprotein fractions. The baseline class separation of cholesteryl esters (CE), cholesterol, triacylglycerols (TG), regioisomers of 1,2- and 1,3-diacylglycerols (DG) and 1-monoacylglycerols (1-MG) is achieved using an optimized hexane - 2-propanol–acetonitrile mobile phase within 18 min for all nonpolar lipid classes or only 9 min excluding monoacylglycerols not detected in studied samples. The determination of individual nonpolar lipid classes is performed by the response factor approach and the use of dioleoyl ethylene glycol as a single internal standard. Polar lipid classes, such as phosphatidylglycerols (PG), phosphatidylethanolamines (PE), phosphatidylcholines (PC), sphingomyelins (SM) and lysophosphatidylcholines (LPC), are separated by hydrophilic interaction liquid chromatography (HILIC) using 5 mmol/L aqueous ammonium acetate–methanol–acetonitrile gradient within 13 minutes. The quantitation of polar lipid classes is done by a similar approach as for nonpolar lipid classes, but a different internal standard (sphingosyl PE d17:1/12:0) is used. The complementary information on fatty acyl profiles after the transesterification of the total lipid extract is obtained by gas chromatography with flame ionization detection (GC/FID). The applicability of developed methodology for fast and comprehensive characterization of blood lipidome is illustrated on samples of human plasma, erythrocytes, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) fractions.

1. Introduction

Lipids are hydrophobic or amphipathic small molecules that may originate entirely or in part by carbanion-based condensations of thioesters and/or by carbocation-based condensations of isoprene units [1,2]. Lipids can be divided into eight basic groups according to the Lipid MAPS consortium: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides [1,3,4]. The division for polar (mainly glycerophospholipids, sphingolipids and saccharolipids) and nonpolar (glycerolipids, sterol lipids and fatty acyls) lipids is frequently used in the analytical chemistry [5], because polar and nonpolar lipids require different procedures for their sample preparation, chromatographic separation and mass spectrometric detection. Lipids are transported throughout the human body using lipoprotein particles, which can be divided according to their relative densities into chylomicrons, VLDL, LDL and HDL [5]. The disruption of lipid synthesis and processing can be correlated with serious human diseases, such as obesity, diabetes, cancer or cardiovascular diseases (CVD) [6,7]. Risk factors of CVD are elevated levels of total cholesterol, LDL cholesterol, triacylglycerols (TG), decreased HDL cholesterol [8,9], obesity, hypertension and hyperglycemia [10].

Chromatographic techniques coupled to mass spectrometry (MS) are typically used for a comprehensive lipidomic analysis of biological samples. Thin-layer chromatography (TLC) is a simple

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and cheap separation method, which can provide the basic information on phospholipid, glycerolipid and sphingolipid classes, but it can be also coupled with matrix-assisted laser desorption/ionization (MALDI) to obtain detailed information on lipid molecular species [11,12]. The routine analytical method for a fatty acyl profiling after the transesterification to fatty acid methyl esters (FAME) is GC/FID or GC/MS [13–16]. High-performance liquid chromatography (HPLC) enables the separation of individual polar lipid classes using HILIC [17–24] or nonpolar lipid classes using NP HPLC [22,24–26]. Reversed-phase (RP) HPLC [18,20,27–30] systems are frequently used for the separation of individual polar lipid species inside lipid classes, while nonaqueous reversed-phase (NARP) HPLC [14,31–33] is preferred for nonpolar lipid species, such as TG. At present, ultrahigh-performance liquid chromatography (UHPLC) offers significant advantages over conventional HPLC, such as the reduction of analysis time, the sensitivity increase and the reduced solvent consumption [34]. Electrospray ionization (ESI) is the most widespread ionization technique used in the MS analysis of polar lipids, while atmospheric pressure chemical ionization (APCI) is more convenient for nonpolar lipids. The quantitation of lipids using MS can be performed by several approaches, e.g., the shotgun approach [35–38] using precursor ion, neutral loss and selected reaction monitoring scans on triple quadrupole or hydride quadrupole–linear ion trap mass spectrometers, HPLC/MS class quantitation using the combination of single internal standard (IS) and response factors (RF) for individual lipid classes related to this IS [39] or HPLC/MS using IS per each lipid class [6].

The main goal of this work is the development of high-throughput methods for the comprehensive lipidomic analysis of blood fractions. We describe the novel NP-UHPLC/APCI-MS method for the quantitation of nonpolar lipid classes and improved HILIC-UHPLC/ESI-MS method for the quantitation of polar lipid classes with good repeatability of retention times and peak areas, which is an essential requirement for the reliable quantitation. The quantitation is performed using IS and RF approach. This methodology may provide the quantitative information on six nonpolar and five polar lipid classes together with the possibility of species quantitation based on mass spectra of peaks of individual lipid classes, which is applicable in the biomarker discovery research of serious human diseases related to lipids.

2. Experimental

2.1. Materials

Acetonitrile, 2-propanol, methanol (all HPLC/MS grade), chloroform stabilized with 0.5–1% ethanol, hexane (both HPLC grade) and ammonium acetate, sodium chloride and sodium methoxide were purchased from Sigma–Aldrich (St. Louis, MO, USA). Deionized water was prepared with Demiwa 5-roi purification system (Watek, Ledeč nad Sázavou, Czech Republic) and by ultra CLEAR UV apparatus (SG, Hamburg, Germany). Standards of CE, TG, 1,2-DG, 1,3-DG and 1-MG containing oleoyl acyls and nonadecanoyl acyls and cholesterol were purchased from Sigma-Aldrich. Standards of polar lipids containing oleoyl acyls (PG, PE, PC, SM and LPC), the IS for nonpolar lipids (dioleoyl ethylene glycol, IS_1), the IS for polar lipids (sphingosyl PE d17:1/12:0, IS_2) and deuterated cholesterol-d7 were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Biological samples (plasma, erythrocytes, VLDL, LDL and HDL) were obtained from healthy volunteers and CVD patients in the cooperation with the University Hospital Olomouc. The experimental setup was approved by the Ethical committee at the University Hospital Olomouc.

2.2. Sample preparation

Blood of healthy volunteer (man, age group 40–55 years, body mass index 25.7) was collected to heparin-lithium tubes and centrifuged to obtain erythrocytes and plasma. Plasma was further separated into lipoprotein fractions by the ultracentrifugation. Nonpolar lipids were extracted according to the modification of previously published procedure [40]: 50 μL of the plasma sample and 5 μL of IS_1 (3.325 mg/mL) were mixed with 150 μL hexane–methanol (98:2, v/v). The mixture was incubated for 10 min, then 300 μL of methanol–water mixture (95:5, v/v) and 300 μL of hexane–methanol mixture (98:2, v/v) were added. The sample was centrifuged for one minute at 25 rpm, and the upper hexane layer was collected. The lower layer was washed twice with 300 μL of hexane–methanol (98:2, v/v), and the upper hexane layer was collected. The combined hexane extract containing nonpolar lipids was evaporated by a gentle stream of nitrogen, then redissolved in hexane and used for NP-UHPLC/APCI-MS analysis.

Total lipid extracts were prepared according to the modified Folch procedure [39,41]. 500 μL of the plasma sample (applicable for the plasma volume at least from 50 to 500 μL) and 50 μl of IS_2 (3.333 mg/mL) were homogenized with 10 mL of chloroform–methanol mixture (2:1, v/v). This mixture was filtered using a rough filter paper. Then 2 mL of 1 mol/L NaCl was added and centrifuged for 3 minutes at 25 rpm. The chloroform layer containing lipids was evaporated by the gentle stream of nitrogen and dissolved in chloroform–2-propanol (1:1, v/v) for the HILIC-UHPLC/ESI-MS analysis. FAME were prepared according to the procedure with sodium methoxide [14,42]. 100 μL of the sample (plasma, erythrocytes or lipoprotein fractions) and 1.6 mL of 0.25 mol/L sodium methoxide in methanol were heated on a water bath for 10 min at 65 °C. 1 mL of 1 mol/L NaCl and 1 mL of hexane were added after the reaction. The upper hexane layer containing FAME was used directly for the GC/FID analysis.

2.3. NP-UHPLC/APCI-MS conditions

Experiments were performed with a liquid chromatograph Agilent 1200 infinity series (Agilent Technologies, Santa Clara, CA, USA). Acquity UPLC HILIC column (50 × 2.1 mm, 1.7 μm, Waters, Milford, MA, USA) was used for the separation of nonpolar classes of lipids (CE, TG, DG, cholesterol and MG). The flow rate was 1 mL/min, the column temperature 30 °C, the mobile phase gradient 0 min – 99% A + 1% B, 20 min – 32% A + 68% B, where A is hexane and B is a mixture of hexane-2-propanol–acetonitrile (96:2:2, v/v/v). Esquire 3000 ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) operating in the positive-ion APCI mode was used for the detection of nonpolar lipids under the following conditions: mass range *m/z* 50–1000, corona voltage 4 μA, pressure of nebulizer gas 65 psi, flow rate of drying gas 3 L/min, drying temperature 350 °C and vaporizer temperature 375 °C. Nonpolar lipids were identified based on *m/z* values of $[M+H]^+$ ions and characteristic fragment ions and also retention times of individual compounds.

2.4. HILIC-UHPLC/ESI-MS conditions

Experiments were performed with a liquid chromatograph Agilent 1290 infinity series (Agilent Technologies). Acquity UPLC HILIC column (50 × 2.1 mm, 1.7 μm, Waters) was used for the separation of polar lipid classes (PG, PE, PC, SM and LPC). The flow rate was 0.5 mL/min, the column temperature was 40 °C, the mobile phase gradient 0 min – 0.5% A + 99.5% B, 20 min 20.5% A + 79.5% B, where A is the mixture of 5 mmol/L aqueous ammonium acetate - methanol (9:1, v/v) and B is acetonitrile. Hybrid quadrupole-time-of-flight (Q-TOF) micrOTOF-Q mass spectrometer (Bruker Daltonics) operating in the positive-ion ESI mode was used for the detection of polar

lipids under the following conditions: mass range *m/z* 50–1500, capillary voltage 4.5 kV, pressure of nebulizer gas 1.6 bar, flow rate of drying gas 9 mL/min and drying temperature 220 °C. Polar lipids were identified based on accurate *m/z* values of $[M+H]^+$ ions and characteristic fragment ions and also retention times of individual compounds.

2.5. GC/FID conditions

GC/FID experiments were performed on the gas chromatograph Agilent 7890 (Agilent Technologies) using TR-FAME column (60 m length, 0.25 mm ID, 0.25 μm film thickness, Thermo Scientific, Waltham, MA, USA) under the following conditions [18]: the injection volume 3 μL, the split ratio 1:15, the flow rate of helium as a carrier gas 1.025 mL/min. The temperature program was the following: the initial temperature 160 °C, ramp to 210 °C at 2 °C/min, ramp to 235 °C at 22 °C/min. Injector and detector temperatures were 250 °C and 280 °C, respectively.

3. Results and discussion

3.1. Sample preparation

The sample preparation is a rather important step in the lipidomic analysis, because inappropriate extraction procedures can cause lower recoveries for some lipid classes and systematic errors in the quantitative analysis. The essential requirement for any LC/MS quantitative assay is the use of appropriate IS for the quantitation. The use of isotopically labeled IS is the best quantitation approach, but it is not applicable for the lipidomic analysis due to the large range of analyzed molecules and the lack of such standards. In the present work, we follow the strategy developed in our previous work [39] based on the use of RF for individual lipid classes combined with the single IS well separated from other lipid classes and not occurring in biological samples.

The new method for the extraction of nonpolar lipids is optimized based on two phase liquid-liquid system with hexane–methanol–water (final conditions in Section 2.2 Sample preparation), where nonpolar lipids are concentrated in the upper hexane layer. After the collection of upper hexane layer, the bottom aqueous methanol layer is washed two times with hexane–methanol mixture (98:2, v/v) to increase the extraction efficiency. Combined hexane extracts are evaporated to dryness, then redissolved in pure hexane and used for further NP-UHPLC/APCI-MS analysis. Extraction recoveries for representatives of nonpolar lipid classes (CE 19:0, TG 19:0/19:0/19:0, cholesterol-d7 and DG 19:0/0:0/19:0) and IS_1 are shown in Table S1. Extraction recoveries for the stock solution without any matrix are in the range 97–110% and for the plasma matrix 93–100%, which clearly confirm the applicability of this method for the real analysis of body fluids without negative matrix effects. The extraction recovery of spiked samples cannot be determined for CE due to the common fragment ion *m/z* 369 corresponding to the loss of fatty acyl chain from CE, which is observed for both lipid class standard of CE and CE in plasma.

The extraction method for polar lipids is used according to previously developed procedure [18,39] based on the modified Folch method (details in Experimental, section 2.2 Sample preparation).

3.2. NP-UHPLC/APCI-MS of nonpolar lipid classes

The class separation of nonpolar lipids cannot be achieved under HILIC conditions, because nonpolar lipids elute unresolved in the void volume of the system [20] and the systematic optimization of separation does not help to increase their retention. For this reason, we have decided to develop new NP-UHPLC/APCI-MS method for

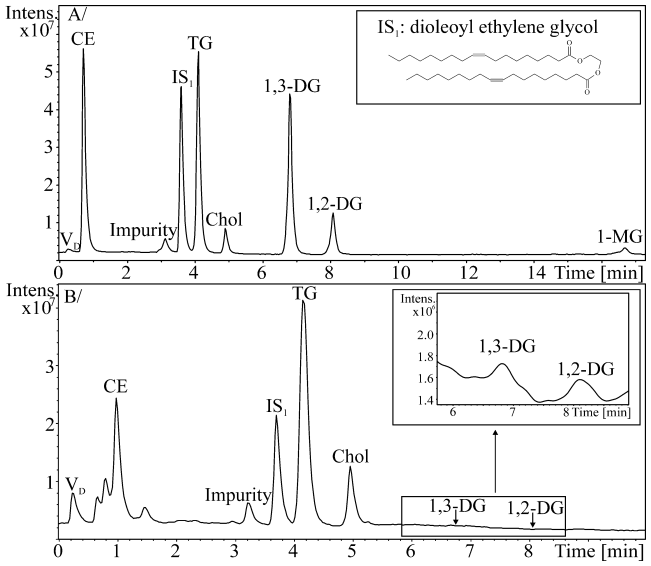

Fig. 1. NP-UHPLC/APCI-MS chromatogram of the separation of nonpolar lipid classes (CE, TG, cholesterol, 1,3-DG, 1,2-DG and 1-MG) and the internal standard IS_1 (dioleoyl ethylene glycol): (A) lipid class standards containing oleoyl acyls and cholesterol, (B) human plasma sample. Conditions: Acquity UPLC HILIC column (50 × 2.1 mm, 1.7 μm), flow rate 1 mL/min, column temperature 30 °C, gradient 0 min – 99% A + 1% B, 20 min – 32% A + 68% B, where A is hexane and B is a mixture of hexane-2-propanol–acetonitrile (96:2:2, v/v/v), positive-ion APCI-MS detection. Peak annotation: CE–cholesteryl esters; TG–triacylglycerols; Chol–cholesterol; 1,3-DG–1,3-diacylglycerols; 1,2-DG - 1,2-diacylglycerols; 1-MG - 1-monoacylglycerols.

the class separation and quantitation of main nonpolar lipid classes (TG, DG, MG, CE and cholesterol) in the shortest possible time suitable for high-throughput clinical analysis of blood samples. The appropriate IS has to be selected before the optimization of separation conditions, because the IS must be well separated from all lipid classes, it must be absent in analyzed samples and exhibit structural similarity with determined classes. Dioleoyl ethylene glycol is selected as the IS for the determination of nonpolar lipids (IS_1), because it fulfills all above mentioned criteria and moreover it is relatively cheap.

The first experiments have been performed on 150 × 2.1 mm silica column packed with 3 μm particles, but obtained results are unsatisfactory due to wider peaks, longer analysis times, low retention of CE and the coelution of IS_1, TG and the peak of impurity from hexane (antioxidant Irgafos 168 providing a signal at *m/z* 663). The column packed with sub-2 μm particles (Acquity UPLC HILIC column, 150 × 2.1 mm, 1.7 μm particles) provides narrower peaks (Fig. S1A), but the retention of CE is still too close to the system void volume and the critical pair of IS_1/TG is not resolved using a generic gradient of 2-propanol in hexane from 5% to 100% B within 30 minutes. A good separation of DG regioisomers, MG and cholesterol can be achieved relatively easily at a wide range of chromatographic conditions. The increased retention of CE further away from V_D can be achieved by decreased initial percentage of 2-propanol as the stronger solvent, but it does not help with the resolution of TG/IS_1 pair (Fig. S1B). Combined effects of the addition of acetonitrile into the polar modifier (2-propanol/acetonitrile, 1:1, v/v) and lowering the gradient steepness lead to the resolution of this critical pair. Increased temperature from 20 °C to 30 °C slightly increases the retention and improves the reproducibility. The low system back-pressure in NP system allows increasing the flow rate from the initial 0.4 mL/min (Figs. S1A and B) through 0.6 mL/min (Fig. S1C) to the final value of 1.0 mL/min (Fig. 1) without the negative effect on the system performance and the back-pressure is still below 200 bars. The final method (Fig. 1A) allows the baseline separation of all nonpolar lipid classes within 18 min or excluding MG

Table 1
Parameters of calibration curves, response factors and the repeatability of measurements for nonpolar and polar lipid class representatives containing oleoyl residues and for internal standards.

Lipid class	Retention time [min]	Slope	Intercept	Regression coefficient	Response factor	Repeatability [%] [a]	
						Retention times	Peak areas
Nonpolar lipids							
CE	0.74	4.519	75.676	0.999	1.06	1.1	3.8
IS_1 [b]	3.59	4.804	13.853	0.999	1.00	0.1	1.3
TG	4.12	6.005	45.665	0.996	0.80	0.4	0.5
Chol	4.88	0.903	−0.113	0.999	5.32	0.2	4.1
1,3-DG	6.81	6.102	10.195	1.000	0.79	0.1	2.3
1,2-DG	8.07	4.447	0.842	1.000	1.08	0.1	4.7
MG	16.65	0.463	−0.324	1.000	10.38	0.1	4.7
Polar lipids							
PG	4.34	0.249	2.569	0.982	2.80	0.2	1.1
PE	7.94	0.699	7.113	0.999	1.00	0.1	0.5
IS_2 [c]	9.63	0.697	7.685	1.000	1.00	0.1	1.6
PC	10.77	0.570	2.788	1.000	1.22	0.1	3.1
SM	11.86	0.293	2.092	0.999	2.38	0.1	4.1
LPC	12.49	0.313	0.676	0.999	2.23	0.1	1.0

[a] Standard deviations based on five consecutive measurements.
[b] Dioleoyl ethylene glycol is used as the internal standard for the determination of nonpolar lipids (IS_1).
[c] Sphingosyl PE d17:1/12:0 is used as the internal standard for the determination of polar lipids (IS_2).

in 9 min only. MG are not detected in studied blood samples, so the final method has been shortened excluding MG class. Fig. 1B illustrates the separation of nonpolar lipids in human plasma sample with abundant presence of CE, TG and cholesterol. 1,3- and 1,2-DG regioisomers are present only at the trace concentration. TG and DG elute in single peaks without a visible resolution of individual molecules within these lipid classes, while individual CE are partially separated (Fig. 1B).

The quantitation is based on the same strategy published recently for polar lipid classes [39] using the RF for individual lipid classes and the single IS. Calibration curves for lipid class representative standards containing oleoyl acyls and IS_1 have been measured. Ratios of slopes of calibration curves for IS_1 over lipid class representative is used for the determination of RF of particular lipid class (Table 1) based on the assumption that differences of relative responses of lipids within one class are small. The same assumption is used in the majority of quantitative approaches both for LC/MS and shotgun lipidomics due to the lack of quantitative lipidomic standards or their high prices. Individual lipoprotein fractions of human plasma and erythrocytes exhibit rather large differences in concentrations of nonpolar lipid classes, as illustrated in Fig. 2 and numerical values are listed in Table 2. Plasma shows similar concentrations of TG, CE and cholesterol, while only cholesterol is detected in erythrocytes. Large differences are found among particular plasma lipoprotein fractions. Some classes are more abundant in LDL compared to VLDL (e.g., CE almost 10 times and cholesterol 4 times), while it is vice versa for DG (e.g., 1,2-DG have almost 10 times higher concentration in VLDL compared to LDL). These concentration differences are in accordance with known metabolic functions of individual lipoprotein fractions [3,5].

Concentrations of individual lipid species within classes can be determined as well (Table S2) based on the assumption used in the majority of lipidomic quantitation that differences in RF within the class can be neglected. Then the concentration of lipid species is calculated by the multiplication of the total lipid class concentration by the relative abundances of individual lipids in the mass spectrum of whole lipid class. Table S2 also shows the comparison of our concentration with previously published work [38] on the NIST human plasma by nine different laboratories within the Lipid MAPS consortium. We obtain a satisfactory correlation despite the simplified way of our quantitation (single IS for all lipid classes instead of IS for each lipid class), different samples of human plasma, different extraction procedures (generic method *vs.* dedicated protocols for individual lipid classes). Over 50% of results in Table S2 differ less than 2 folds, almost 70% of concentrations differ less than 3 folds and only 8 values differ more than ten times, which is acceptable considering all discussed differences. There is also good agreement between identified lipid species in both data sets, while some lipids are not detected in one of these works, typically at low concentration levels. We have a lower number of identified nonpolar lipids due to necessity to use APCI in NP mode, which causes more extensive fragmentation (especially for CE) resulting in less detected intact lipid molecules.

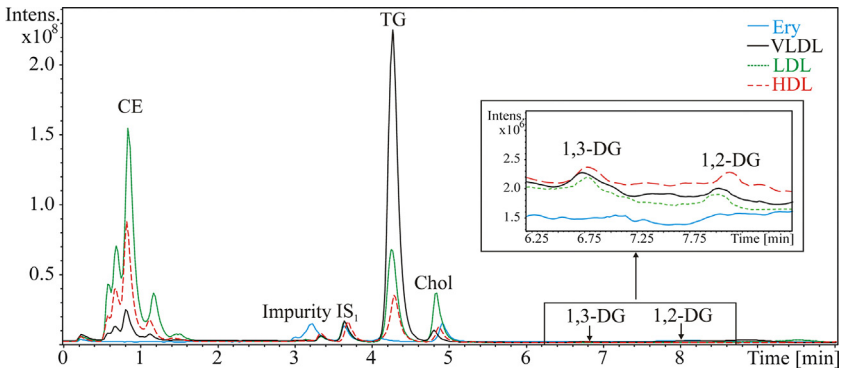

Fig. 2. NP-UHPLC/APCI-MS chromatograms of nonpolar lipid classes in human plasma lipoprotein fractions (HDL, LDL and VLDL) and erythrocytes. NP-UHPLC conditions are identical as for Fig. 1.

Table 2
Retention times and concentrations of nonpolar lipid classes by NP-UHPLC/APCI-MS and polar lipid classes by HILIC-UHPLC/ESI-MS determined in human plasma, erythrocytes and lipoprotein fraction samples.

Lipid class	Concentration [μg/mL]				
	Plasma	Erythrocytes	VLDL	LDL	HDL
Nonpolar lipids					
CE	770.5	n.d.[a]	470.2	4037.4	2180.8
TG	817.9	n.d.[a]	1845.8	1153.2	574.2
Chol	645.4	1946.1	578.7	2458.3	746.3
1,3-DG	2.1	n.d.[a]	8.9	4.0	13.6
1,2-DG	5.2	n.d.[a]	21.9	2.4	11.0
Polar lipids					
PE	60.7	159.3	34.6	48.9	68.4
PC	615.2	262.3	447.8	651.4	709.1
SM	264.4	159.5	123.4	307.9	197.3
LPC	189.5	23.3	22.0	72.1	55.3

[a] n.d. – not detected, below the limit of detection: 0.028 μg/mL for CE, 0.016 μg/mL for TG, 0.11 μg/mL for Chol, 0.015 μg/mL for 1,3-DG and 0.028 μg/mL for 1,2-DG.

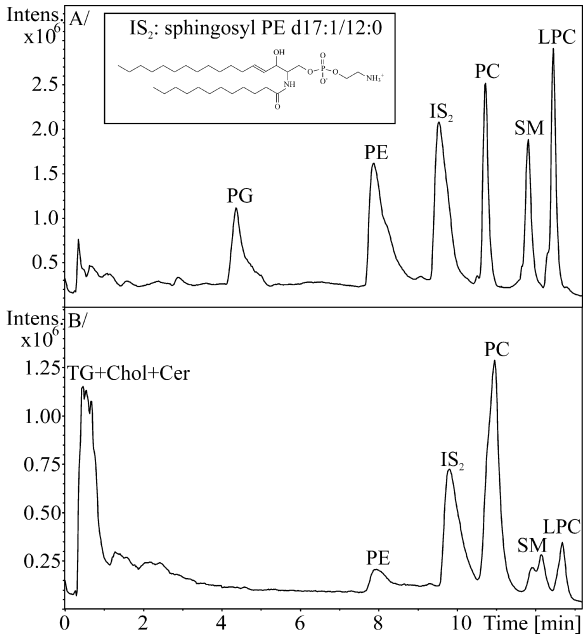

Fig. 3. HILIC-UHPLC/ESI-MS chromatograms of the separation of polar lipid classes (PG, PE, PC, SM and LPC) and the internal standard IS_2 (sphingosyl PE d17:1/12:0): (A) lipid class standards containing oleoyl acyls, and (B) human plasma sample. Conditions: Acquity UPLC HILIC column (50 × 2.1 mm, 1.7 μm), flow rate 0.5 mL/min, column temperature 40 °C, gradient 0 min – 0.5% A + 99.5% B, 20 min 20.5% A + 79.5% B, where A is a mixture of 5 mmol/L aqueous ammonium acetate–methanol (9:1, v/v), and B is acetonitrile, positive-ion ESI-MS detection. Peak annotation: PG–phosphatidylglycerols; PE–phosphatidylethanolamines; PC–phosphatidylcholines; SM–sphingomyelins; LPC–lysophosphatidylcholines.

3.3. HILIC-UHPLC/ESI-MS of polar lipid classes

The method development of polar lipid classes separation has started from our previously published HILIC-HPLC/ESI-MS method for the separation of multiple polar lipid classes [39]. The analysis time of this older method (60 min) is not acceptable for high-throughput clinical analysis and some lipid classes are not present in plasma and erythrocytes, so the optimization of their separation is useless. The first step for the reduction of analysis time is the selection of appropriate particle size and column geometry. The particle size of sub-2 μm and short 50 mm column is selected for the fast separation of all polar lipid classes determined in blood fractions (PE, PC, SM and LPC). The best results are achieved on the identical column as for NP separation (Acquity UPLC HILIC column, 50 × 2.1 mm, 1.7 μm), but one column is used only for nonaqueous NP and the second one for aqueous HILIC systems without changing columns between systems to avoid problems with the column equilibration while changing between nonaqueous and aqueous mobile phase systems. It was reported previously [26] and verified in our preliminary experiments that the use of mobile phase system combining nonaqueous part of gradient for nonpolar lipid classes followed by the aqueous gradient step used for the elution of polar lipid classes results in a lower repeatability of retention times (up to 6%) and peak areas (up to 10.5%) in comparison to separate methods used in this work (Table 1).

Total ion current chromatograms obtained with the final HILIC-UHPLC/ESI-MS method are shown for the mixture of standards of lipid class representatives (Fig. 3A) and the real sample of human plasma (Fig. 3B). The quantitative approach is based on the identical principle as for nonpolar lipids described in the previous chapter. The IS for the polar lipid class determination is sphingosyl PE d17:1/12:0 (IS_2) in accordance with our previous work [39]. Parameters of calibration dependencies with the lipid class RF are listed in Table 1. Differences in polar lipid composition among plasma lipoprotein fractions and erythrocytes (Fig. 4) are lower than in case of nonpolar lipids (Table 2). Only erythrocytes exhibit higher concentrations of PE and lower concentration of PC in comparison to other blood fractions. Concentrations of individual lipid species are determined (see Table S2) the same way for nonpolar lipid classes discussed above.

3.4. GC/FID

GC/FID is well established technique routinely used for the determination of fatty acyl profiles, which provide useful complementary information during the interpretation of mass spectra of

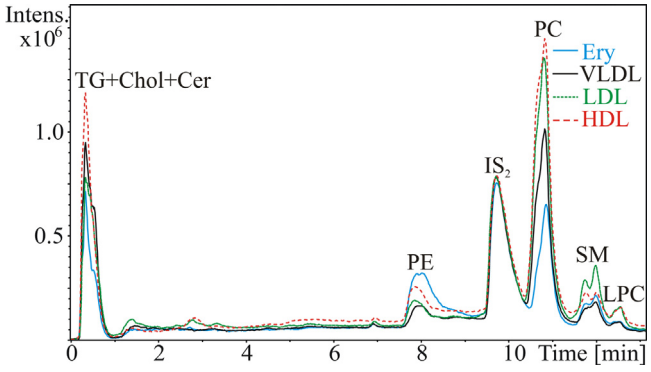

Fig. 4. HILIC-UHPLC/ESI-MS chromatograms of polar lipid classes in human plasma lipoprotein fractions (HDL, LDL and VLDL) and erythrocytes. Conditions are identical as for Fig. 3.

complex lipids. Fig. S3 illustrates the optimized GC separation of FAME in plasma, erythrocytes, VLDL, LDL and HDL of one person together with the quantitative data shown in Table S2.

4. Conclusions

Two complementary methods are developed for the determination of both nonpolar and polar lipid classes detected in human blood samples. NP-UHPLC/APCI-MS is used for five nonpolar lipid classes (CE, cholesterol, TG, 1,2-DG and 1,3-DG), while HILIC-UHPLC/ESI-MS is applied for five polar lipid classes (PG, PE, PC, SM and LPC). Both methods are optimized in terms of good separation of individual lipid classes, method robustness and applicability for high-throughput lipidomic characterization of large series of human plasma, erythrocytes and lipoprotein fraction samples typical for biomarker discovery studies. The quantitation approach is identical for both methods using RF of individual lipid classes, but different IS are used for the determination of nonpolar and polar lipid classes. GC/FID of FAME provides additional information on fatty acyl profiles, which shows differences among individual blood fractions. The applicability of these methods is already verified in the running project devoted to CVD, where hundreds of samples are measured and above mentioned lipid classes are quantified. Concentrations of lipid species inside particular classes are determined from their relative abundances within the class multiplied by the total class concentration and compared with the previously published work on the human plasma [38].

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Appendix A. Supplementary data

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