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Quantification of bioactive sphingo- and glycerophospholipid species by electrospray ionization tandem mass spectrometry in blood☆

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ABSTRACT

Bioactive glycerophospho- and sphingolipids species are involved in the regulation of numerous biological processes and implicated in the pathophysiology of various diseases. Here we review electrospray ionization tandem mass spectrometric (ESI-MS/MS) methods for the analysis of these bioactive lipid species in blood including lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), bis(monoacylglycero)phosphate (BMP), ceramide (Cer), sphingosine-1-phosphate (S1P) and sphingosylphosphorylcholine (SPC). Beside direct tandem mass spectrometric and liquid chromatography coupled approaches, we present an overview of concentrations of these bioactive lipids in plasma. The analytical strategies are discussed together with aspects of sample preparation, quantification and sample stability.

1. Introduction

The discovery of sphingolipids as signalling molecules in the early 1990s [1,2] together with the finding of specific cell surface receptors for lysophospholipids in the late 1990s [3,4] inspired the research in the field of bioactive lipids the past decades. As meanwhile robust and sensitive electrospray ionization tandem mass spectrometers (ESI-MS/MS) are available, this technique may be considered as the method of choice for the analysis of bioactive lipid species. This review provides an overview of ESI-MS/MS methods for the quantification of bioactive glycerophospho- and sphingolipid species and their analysis in blood.

2. Biology and pathobiology of bioactive glycerophospho- and sphingolipid species

2.1. Structures, blood level and sources

Most of lipid species identified as bioactive molecules belong to the lysophopholipids (Fig. 1) including the glycerophospholipids lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC) and the sphingolipids sphingosine-1-phosphate (S1P), sphingosylphosphorylcholine (SPC) [5]. Other examples are the highly hydrophobic ceramide (Cer) or bis(monoacylglycero)phosphate (BMP) with its unique structure (Fig. 1) [6–8].

All these lipids were found in plasma of healthy controls (Table 1). While LPC occurs in high μM concentrations, the other bioactive lipids are present in the μM range or below in plasma (Table 1). The majority of plasma LPC is bound to albumin [9,10]. Other lysophospholipids are mainly found in lipoprotein fractions for instance the majority of plasma S1P was bound to HDL [11–13]. BMP was shown to be associated with approximately 40% in lipoproteins and 60% remained in the lipoprotein-deficient plasma [14]. Main carrier for Cer is LDL [10,13] and no Cer is found in albumin fractions [10]. Interestingly, Cer species profiles show differences between the major lipoprotein fractions [10].

Plasma LPC is generated during cholesterol esterification by lecithin-cholesterol acyltransferase (LCAT) or phospholipase A2 action [15]. Both S1P and LPA are generated during platelet activation [16,17]. Beside stimuli dependent release from platelets, erythrocytes release S1P constitutively. Main source of plasma LPA is the autotaxin mediated degradation of LPC [18]. However, not much is known about the origin of plasma Cer, SPC or BMP.

2.2. Bioactivity of glycerophospho- and sphingolipid species

It is well known that lysophospholipids may act as signalling molecules via G-protein coupled receptors [5,19]. To act as messenger their amphiphilic nature allows presence in aqueous and membranous compartments [20]. This way lysophospholipids are involved in numerous biological processes such as regulation of

Abbreviations: BMP, bis(monoacylglycero)phosphate; Cer, ceramide; ESI-MS/MS, electrospray ionization tandem mass spectrometry; HILIC, hydrophilic interaction chromatography; IS, internal standard; LC, liquid chromatography; LOD, limit of detection; LPA, lysophosphatidic acid; MTBE, methyl tertiary butyl ether; NP, normal phase; PG, phosphatidylglycerol; RP, reversed phase; S1P, sphingosine-1-phosphate; SPC, sphingosylphosphorylcholine.

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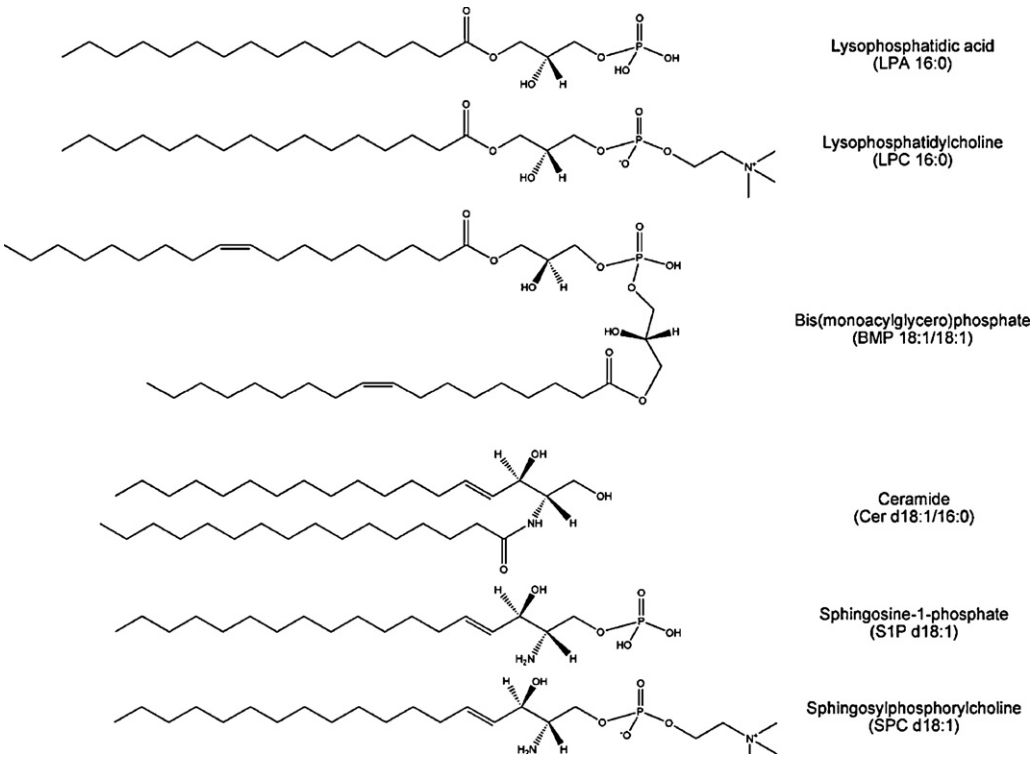

Fig. 1. Chemical structures of bioactive lipids. Representative major lipid species of human plasma are shown with their mass spectrometric shorthand notation in brackets.

Table 1
Concentrations of bioactive lipids in human plasma of healthy controls determined by mass spectrometric analysis. *Abbreviations*: BMP, bis(monoacylglycero)phosphate; Cer, ceramide; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; PA, phosphatidic acid; S1P, sphingosine-1-phosphate; SPC, sphingosylphosphorylcholine (sphingoid bases are abbreviated as number of hydroxyl groups followed by the number of C-atoms and double bonds; d = di).

Lipid class	Material	Concentration	Literature
LPA	EDTA plasma	0.70 ± 0.20 μM	[54]
	EDTA plasma	0.95–2.03 μM (highly acidic extraction)	[81]
	Plasma	4.3–5.1 μM (highly acidic extraction)	[62]
	EDTA plasma	0.61 ± 0.14 μM (male) 0.74 ± 0.17 μM (female)	[82]
	Citrate plasma	2.45 ± 0.74 μM (female)	[83]
LPC	Plasma	103 μM (pooled plasma)	[73]
	EDTA plasma	330 ± 168 μM	[10]
	EDTA plasma	279 ± 7 μM	[30]
	Citrate plasma	243 ± 70 μM (female)	[83]
BMP	Plasma	0.047 ± 0.012 μM	[14]
Cer d18:1	EDTA plasma	5.67 ± 1.45 μM	[13]
	EDTA plasma	5.37 ± 0.55 μM	[84]
	Plasma	6.55 μM (pooled plasma)	[73]
	EDTA plasma	8.1 ± 3.4 μM	[10]
	EDTA plasma	7.6 ± 0.2 μM	[30]
SPC d18:1	EDTA plasma	0.053 ± 0.030 μM	[12]
	Citrate plasma	0.02 ± 0.02 μM (female)	[83]
S1P d18:1	EDTA plasma	0.53 ± 0.09 μM	[12]
	Plasma	0.59 ± 0.04 μM	[85]
	EDTA plasma	0.31 ± 0.05 μM	[13]
	Plasma	0.27–0.85 μM	[86]
	Plasma	0.31 μM (pooled plasma)	[73]
	Plasma	0.70 ± 0.04 μM	[63]
	EDTA plasma	0.47 ± 0.14 μM	[75]
	Citrate plasma	0.58 ± 0.17 μM (female)	[83]
S1P d18:0	EDTA plasma	0.29 ± 0.07 μM	[12]
	Plasma	0.13 ± 0.02 μM	[85]
	EDTA plasma	0.044 ± 0.009 μM	[13]
	Plasma	0.02–0.17 μM	[86]
	Plasma	0.10 μM (pooled plasma)	[73]
	Plasma	0.33 ± 0.03 μM	[63]
	EDTA plasma	0.21 ± 0.06 μM	[75]

cell growth, differentiation immuno-modulation and development [5,18,19,21].

Although it has been reported that Cer may act via receptor binding [22] its main bioactive action may be related to the modulation of membrane properties. Due to its high melting temperature Cer is able to induce the formation of membrane domains [23,24] which are important platforms for signalling, viral infection and membrane trafficking [25]. Moreover, secretory sphingomyelinase mediated generation of Cer induces the aggregation of LDL, a potential cause for subendothelial retention of LDL and atherosclerosis development [26]. The unique chemical structure of BMP and its influence on membrane properties appears to be critical for cholesterol sorting and lysosomal function [27].

Lipid metabolism is a highly dynamic process, which is strictly regulated by a multitude of enzymatic reactions. For example, the conversion of LPC to LPA is just a one-step enzymatic reaction [18]. Bioactive sphingolipids with in part opposite functions like Cer, sphingosine (SPH) and S1P are directly interconnected [6]. Therefore, determination of the lipid pattern, instead of a single metabolite is crucial to get insight into (patho)-physiological processes.

2.3. Bioactive glycerophospho- and sphingolipid species in disease and as biomarker

There exist numerous reports showing that bioactive lipid species play a role in the pathogenesis of various diseases and may be applied as biomarker. For example S1P is suggested to mediate the cardioprotective effect of HDL [28]. In contrast to the protective effect of S1P, plasma Cer were strongly correlated with circulating levels of IL-6 in patients with coronary heart disease [29] and high Cer to sphingomyelin ratios were associated with mortality in sepsis patients [30]. LPA and its precursor LPC were implicated in the progression of atherosclerotic lesions [31]. The LPC content in LDL correlated with lipoprotein-associated phospholipase A2, a predictor for the development of cardiovascular diseases [32].

Furthermore LPC is described as an important immunoregulatory molecule [33]. Low plasma levels of LPC were associated with mortality in patients with sepsis [30]. Interestingly, LPC administration protected mice against lethality after intraperitoneal bacteria infections [34]. S1P regulates lymphocyte egress from lymph node and S1P receptor modulators are in clinical development e.g. for multiple sclerosis [35]. Beside regulation of immune functions bioactive lipids are related to the development of cancer [36,37] and lysophospholipids were described as biomarker for ovarian cancer [38,39] or colorectal cancer [40].

Despite a number of open questions bioactive lipids are implicated in pathogenesis of diabetes [41]. As for instance LDL isolated from diabetics contains an increased LPC content compared to controls [32]. Interestingly, the anti-diabetic drug Metformin decreases LPC in hepatocytes accompanied by a reduced apo B secretion [42] and lower level of LPC were found upon Metformin treatment [43]. Recently, LPA was suggested as mediator of cholestatic pruritus [44]. A potential application as biomarker was shown for BMP which is elevated in lysosomal storage disease [14].

Taken together, all these studies revealed that bioactive lipids are involved in the pathogenesis of various diseases and may be considered for the development of novel biomarker.

3. Sample preparation

Commonly, sample preparation for bioactive lipid analysis is based on liquid–liquid extraction (Table 2). General methods for lipid extraction use chloroform like the procedures according to Folch et al. [45] and Bligh and Dyer [46]. Using chloroform or the recently presented methyl tertiary butyl ether (MTBE) extraction [47] a nearly complete recovery of both, apolar and polar phospholipid species is possible. Therefore, these approaches may be used for bioactive lipids like Cer [48–50] and lysophospholipids like LPC [51–53]. However, highly acidic lysophospholipids like LPA and S1P show a poor recovery in apolar solvents like chloroform without acidification [51]. Addition of strong acids like HCl may lead to a conversion of LPC to LPA in plasma samples with a several-fold artificial increase of LPA [54] (Table 1). A novel two step extraction shows about 60% recovery of LPA and sphingoid bases phosphates in chloroform under slightly basic conditions with ammonium bicarbonate buffer from yeast samples [55].

An alternative to chloroform extractions is a butanolic extraction procedure (pH 4) as described by Baker et al. [56]. Accordingly, Scherer et al. developed methods for the determination of LPA, S1P [54], minor sphingolipids including SPC, free sphingoid bases, hexosyl-, lactosylceramides [12,57] and BMP, PG, cardiolipin, phosphatidic acid [58] from the same butanolic extract [12]. Extraction yields were found to be in the range of 60–95% depending on the polarity of the respective lipid class [12,54,58].

4. Sample material and stability of bioactive lipids in blood

A very important issue is the stability of bioactive lipids in blood. Since bioactive lipids like S1P or LPA are released or generated during blood coagulation plasma, instead of serum, is the material of choice to study bioactive lipids. Hammad and colleagues compared different anticoagulants and their effects on sphingolipid levels in plasma and found EDTA as the most reliable material [13] (the author's lab also uses EDTA plasma as standard material for lipid species analysis [30,59]). Great care is necessary to get reliable results for lysophospholipids. While LPC increases moderately (~25% after 4 h) [52], LPA shows a more than 2-fold rise after 4 h at room temperature in separated plasma [54]. In contrast to LPA, S1P and SPC are stable for at least 24 h at room temperature in plasma [12,54]. In whole blood samples, S1P and LPA show a 2- to 3-fold increase already after 1 h [54] and SPC decreases about 40% after 4 h at room temperature [12]. Taken together, for lysophospholipid analysis whole blood samples have to be centrifuged immediately after sample drawing and the recovered plasma should be frozen instantly.

5. Electrospray ionization tandem mass spectrometry of bioactive lipid species

Due to their polar head groups (Fig. 1) most of the bioactive lipid species are easily accessible by electrospray ionization tandem mass spectrometry (ESI-MS/MS). This technique provides a sensitive and specific platform to quantify bioactive lipid species by two main approaches either direct infusion ("shotgun") or liquid chromatography (LC) coupled analysis. Table 2 presents an overview of existing methods for the analysis of bioactive lipids. We did not include the limits of detection (LODs) in Table 2 since the LOD was reported only for a part of these methods and does not only reflect the sensitivity of the method but also of the instrument used.

5.1. Direct mass spectrometry

Direct analysis of crude lipid extracts is described for Cer [10,48,49], LPC [52,53,60], LPA [60–62] and S1P [63]. Main advantages of direct analysis are the low analysis time, a combined analysis of multiple lipid classes including screening for unknown lipid species [64,65]. Moreover, co-elution of internal standards, an important issue concerning quantification, is an inherent feature of this approach. Since suppression of ionization by matrix components have to be considered in particular for direct MS approaches, application of two internal standards (IS) allow a response control of individual samples [52,66,67]. Matrix effects also limit the sensitivity of direct infusion analysis due to signal suppression at high sample concentrations [68]. Furthermore, results from direct MS/MS analysis may be impaired by the presence of other isobaric compounds (e.g. differentiation of BMP from phosphatidylglycerol [58]) or by application of unspecific mass transitions (e.g. loss of water). A promising alternative approach to resolve quasi-isobaric species (e.g. ether and ester species) is high resolution MS [69]. Accurate mass determination of intact precursors reveals sufficient selectivity to identify and quantify lipid species without fragmentation. This strategy was applied already for the quantification of LPC in plasma [70] and of lysophospholipids and sphingoid bases phosphates in yeast [55]. In-source fragmentation of LPC [71] and lysophosphatidylserine (LPS) [51,71] to LPA may interfere the direct flow injection analysis of LPA from plasma samples. In summary, direct mass spectrometric analysis is confined to lipid species occurring at μM concentrations in plasma (Table 1) showing a specific fragment ion (Table 2), as for example LPC and Cer.

5.2. LC–MS/MS

Interfacing MS/MS with LC offers the following advantages compared to direct infusion analysis: separation of analytes reduces matrix effects and subsequently allows higher sample concentrations resulting in increased sensitivity at the low nmolar range (Table 1). LC is used to separate isobaric compounds [58] as well as quasi-isobaric compounds which may result from different bond types (ester/ether) [69,72].

In contrast to direct MS, small time windows of the analyte peaks usually do not allow untargeted analysis of lipid species. The selection of appropriate separation conditions and internal standards (IS) is of great importance since only co-elution of analyte and IS allows accurate compensation of matrix effects and consequently a reliable quantification. Usually, fatty acids linked to the glycerol or sphingosine backbone vary between 14 and 24 carbon atoms

Table 2
Methods for the tandem mass spectrometric analysis of bioactive lipid species. *Abbreviations* (see Table 1): C1P, ceramide-1-phosphate; CL, cardiolipin; HexCer, Hexosylceramide; LacCer, Lactosylceramide; PA, phosphatidic acid; LPE, lysophoshatidylethanolamine; LPG, lysophoshatidylglycerol; LPI, lysophoshatidylinositol; LPS, lysophoshatidylserine; PG, phosphatidylglycerol; SPH, free sphingoid bases; n.s., not specified.

Analytes	Authors	Sample volume (μL)	Sample preparation	LC type	Analysis time (min)	MS mode transitions fragment ions	Internal standard(s)	Co-elution analyte/IS
BMP	Meikle et al. [14]	100	Liquid–liquid extraction	RP C18	5	ESI+, NL MAG	BMP 14:0/14:0	No
BMP, CL, PA, PG	Scherer et al. [58]	75 (unpublished data)	Liquid–liquid extraction	HILIC	4.5	BMP, ESI+, NL MAG	BMP 14:0/14:0	Yes
Cer	Han [48]	n.s.	Liquid–liquid extraction	Direct infusion	10	ESI−, NL 256	Cer d18:1/17:0	–
Cer	Kasumov et al. [84]	50	Liquid–liquid extraction SPE	RP C8	21	ESI+, *m/z* 264	Cer d18:1/17:0 Cer d18:1/25:0	No
Cer	Liebisch et al. [10,49]	20	Liquid–liquid extraction	Direct flow injection	1.3	ESI+, *m/z* 264	Cer d18:1/14:0 Cer d18:1/17:0	–
Cer,S1P	Shaner et al. [50]; Quehenberger et al. [73]	10	Hydrolysis, Liquid–liquid extraction	Cer – NP-NH_2 S1P – RP C18	Cer – 7 S1P – 8.1	ESI+, *m/z* 264	Cer d18:1/17:0 S1P d17:1	Cer – yes S1P – no
Cer, S1P, SPH	Bielawski et al. [87] Hammad et al. [13]	100	Liquid–liquid extraction	RP C8	30	Cer, S1P, ESI+, *m/z* 264	S1P d17:1 Cer d18:1/17:0 Cer d13:1/16:0 Cer d17:1/16:0 Cer d17:1/24:1	No
LPA	Baker et al. [82]	450	Liquid–liquid extraction	NP Si	>10	ESI−, $[M-H]^-$	$[D_{35}]$-LPA 18:0	Yes
LPA	Ishida et al. [61]	100	SPE	Direct flow injection	n.s.	ESI−, *m/z* 153	LPE 14:0	–
LPA	Shan et al. [81]	500	Liquid–liquid extraction	RP C18	8	ESI−, *m/z* 79, 153	$[^{13}C_{16}]$-LPA 16:0	Only LPA 16:0
LPA	Yoon et al. [62]	200	Liquid–liquid extraction	Direct flow injection	1.5	ESI−, *m/z* 79	LPA 14:0	–
LPA, LPC, LPE, LPG, LPI, LPS	Bollinger et al. [51]	100	Liquid–liquid extraction	NP-Si	52	LPC ESI+, *m/z* 184 LPA ESI−, *m/z* 153	$[D_{31}]$-LPLs 16:0	Partially yes
LPA, LPC, LPI, LPS	Xiao et al. [60]	1000	Liquid–liquid extraction, TLC	Direct flow injection	n.s.	LPA ESI−, *m/z* 79 LPC ESI+, *m/z* 184	LPA 17:0	–
LPA, LPC, S1P, SPC	Murph et al. [83]	500	Liquid–liquid extraction	LPA: RP C5 LPC: RP 18	15	LPC, SPC, ESI+, *m/z* 184 LPA, ESI−, *m/z* 153 S1P, ESI−, *m/z* 79,	LPA 17:0 LPC 17:0	No
LPA, S1P	Scherer et al. [54]	75	Liquid–liquid extraction	HILIC	2.5	S1P, ESI−, *m/z* 79 LPA, ESI−, *m/z* 153	$[^{13}C_2D_2]$-S1P d18:1, LPA 17:0	Yes
LPC	Liebisch et al. [52,53]	20	Liquid–liquid extraction	Direct flow injection	1.3	ESI+, *m/z* 184	LPC 13:0 LPC 19:0	–
LPC	Takatera et al. [88]	20	Liquid–liquid extraction SPE	NP-NH_2	12	ESI+, *m/z* 184	$[D_{35}]$-LPC 18:0	Yes
S1P	Berdyshev et al. [85]	100	Liquid–liquid extraction Acetylation	RP C8	12	ESI−, NL 60	S1P d17:1	No
S1P	Cutignano et al. [75]	1000	Liquid–liquid extraction	UPLC HILIC	20	ESI−, *m/z* 79	S1P d17:1	Yes
S1P	Jiang and Han [63]	100	Liquid–liquid extraction	Direct infusion	1	ESI−, *m/z* 79	S1P d17:1	–
S1P	Schmidt et al. [86]	n.s.	Protein precipitation	RP C18	14.5	ESI+, *m/z* 264	S1P d17:1	No
S1P, SPH	Lan et al. [89]	25	Protein precipitation	RP C18	4	S1P, ESI+, *m/z* 264	S1P d17:1	No
SPC, SPH, C1P, HexCer, LacCer	Scherer et al. [12,57]	75	Liquid–liquid extraction	HILIC	4.5	SPC, ESI+, *m/z* 184	SPC d17:1	Yes

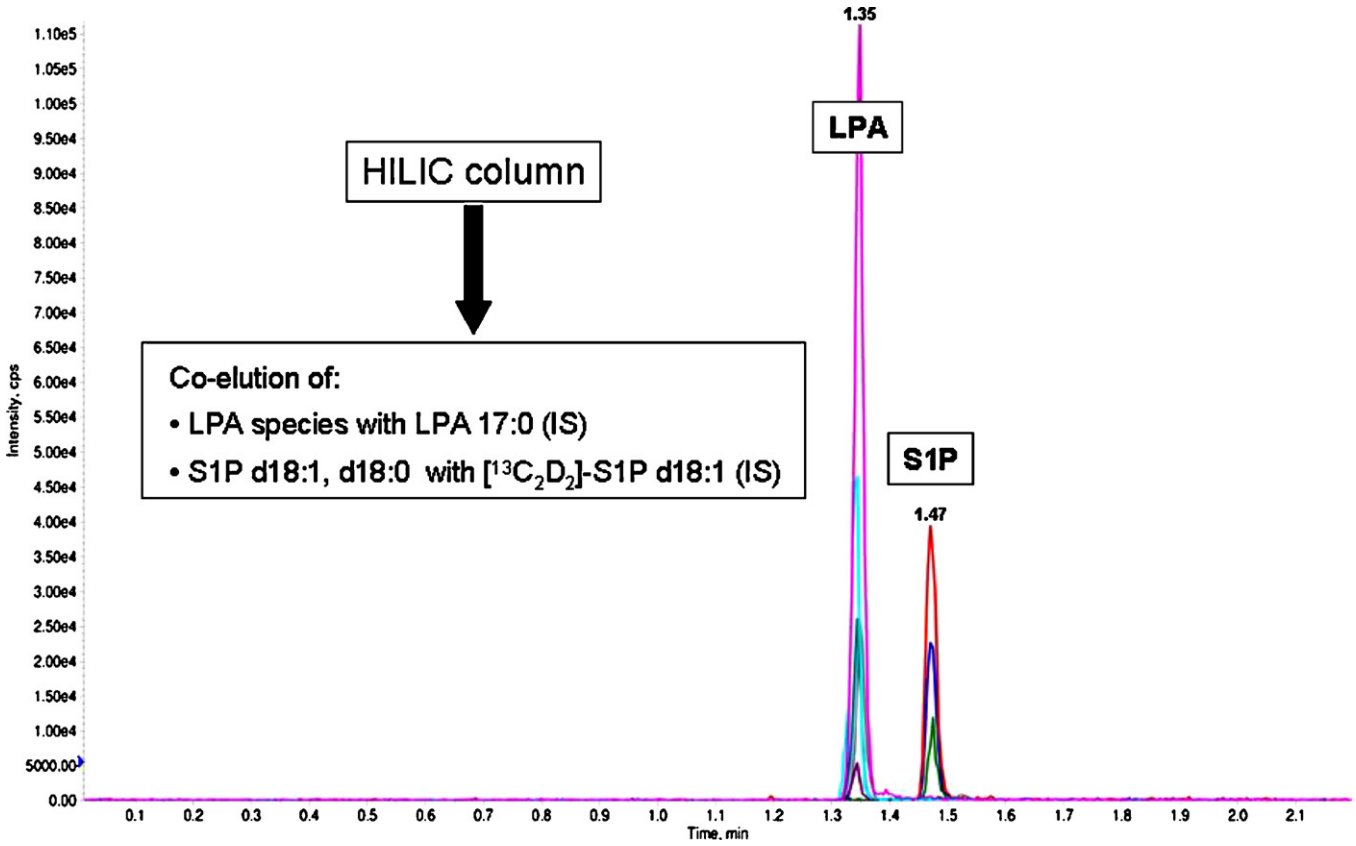

Fig. 2. Chromatogram of LPA (natural LPA species and LPA 17:0) and S1P (S1P d18:1, d18:0 and [$^{13}C_2D_2$]-S1P d18:1) analysis by HILIC–MS/MS from a human plasma sample [12,54]. 75 μl of human EDTA plasma was extracted with 1-butanol in the presence of the internal standards LPA 17:0 and [$^{13}C_2D_2$]-S1P d18:1. Butanolic extracts were analyzed by hydrophilic-interaction chromatography (50 × 2.1 mm, 2.2 μm particle size, Interchim, Montlucan, France) tandem mass spectrometry with a run time of 2.5 min. LPA and S1P species were detected in negative electrospray ionization mode using *m/z* 153 and 79 fragment ions, respectively.

with 0–6 double bonds in human plasma [10,12,13,52,54,58,73,74]. There are two general LC strategies – normal and reversed phase chromatography. Normal phase (NP) chromatography shows selectivity towards polar lipid head groups while reversed phase (RP) has an affinity to apolar moieties of lipid species. RP chromatography usually separates lipid species from one lipid class according to the fatty acid chains. Since stable isotope labeled internal standards are usually not commercially available, frequently medium-chain non-naturally occurring lipid species were used as ISs (Table 2). Using medium-chain ISs, quantification by RP LC–MS/MS may be affected by matrix effects since analytes and ISs do not co-elute (Table 2). NP chromatography allows co-elution of analytes and ISs but may exhibit a low reproducibility. Recently, LC–MS/MS methods were presented for the quantification of S1P [54,75], LPA [54] (Fig. 2), SPC [12] and BMP [58] based on hydrophilic interaction chromatography (HILIC). HILIC provides a polar selectivity leading to co-elution of lipid classes with their respective ISs (Fig. 2). Compared to classical NP, HILIC shows advantages in terms of reproducibility, peak performance and an easy change of the LC system to RP methods (by avoiding apolar solvent mixtures containing hexan, chloroform).

5.3. Quantification

Most important for accurate quantification is the inclusion of appropriate IS into the analysis (Table 2). At least one IS should be included per lipid class. Two IS per lipid class allow a quality check for individual samples which is beneficial especially for direct MS/MS methods [52,66]. The analytical response of respective lipid species may greatly depend on structural details. For example in comparison to d18:0, S1P d18:1 exhibits a 3-fold higher response and polyunsaturated LPA 20:4 a 75% lower response compared to other LPA species [54]. Therefore, it is recommended to use a variety of lipid species for calibration to address these differences. Calibration by standard addition to plasma should be preferred, since it allows a compensation of potential matrix effects on analytical response or extraction recovery. The use of charcoal treated plasma as an analyte free sample matrix is not recommended since charcoal may remove also other lipids responsible for analytical interferences present in untreated samples.

6. Conclusions and outlook

Due to their important regulatory functions the analysis of bioactive lipid species may improve our understanding of a number of different diseases. Moreover, these lipids have the potential to be used as biomarkers in these disorders. Although the sensitivity of the current LC–MS/MS methods is sufficient for most of the lipids described above, minor species like sphingolipids with bases other than sphingosine (d18:1) are still close to the limit of detection [12].

To get a more comprehensive picture of the species variety the next generation of mass spectrometers with enhanced sensitivity should be of great help. This way lipid species profiles from lipoprotein fractions [74], blood cells [76] and tissues signatures [77] may be expanded and could help to understand the species heterogeneity and origin of bioactive lipid species. Furthermore, metabolic profiling of the lipid metabolism with stable isotope labeled precursors could be a valuable tool to substantiate this knowledge [78–80].

A key challenge for studying bioactive lipids like lysophospholipids is a valid pre-analysis. In order to get correct in vivo level of

these lipids it is necessary to stabilize the samples already during blood drawing. Potential solutions are phospholipase inhibitors or platelet stabilizing agents which are added to the blood collection tube.

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