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Simple and Robust Measurement of Blood Plasma Lysophospholipids Using Liquid Chromatography Mass Spectrometry

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Abstract : Single analytical procedure including extraction, liquid chromatography, and mass spectrometric analysis was evaluated for the simultaneous measurement of lysophospholipids (LPLs). LPLs, particularly, lysophosphatidic acids (LPA) and sphingosine 1-phosphate (S1P) are lipid messengers ubiquitously found in various biological matrix. The molecular species mediate important physiological roles in association with many diseases (e.g. cancer, inflammation, and neurodegenerative disease), which emphasize the significance of the simple and reliable analytical method for biomarker discovery and molecular mechanistic understanding. Thus, we developed analytical method mainly focusing on, but not limited by those lipid species S1P and LPA using reverse phase liquid chromatography-tandem mass spectrometry (RPLC-ESI-MS-MS). Extraction method was modified based on Folch method with optimally minimal level of ionization additive (ammonium formate 10 mM and formic acid). Reverse-phase liquid-chromatography was applied for chromatographical separation in combination with negative ionization mode electrospray-coupled Orbitrap mass spectrometry. The method validation was performed on human blood plasma in a non-targeted lipid profiling manner with full-scan MS mode and data-dependent MS/MS. The proposed method presented good inter-assay precision for primary targets, S1P and LPA. Subsequent analysis of other types of LPLs identified a broad range of lysophosphatidylcholines (LPCs) and lysophosphatidyl-ethanolamines (LPEs).

Keywords : Lysophospholipid, LC-MS/MS, lipidomics, lysophosphatidic acid, sphingosine 1-phosphate

Introduction

Phospholipids (PLs) and lysophospholipids (LPLs) are main classes of cell membrane and also key molecules in biological function such as cell proliferation and death.[1]

Accordingly, the molecular species have recently been found closely implicated with biomarkers and potential therapeutic targets for various types of human diseases.[2,3] S1P and LPA (C18:1) have been found to be important signaling molecules[4,5] and potential molecular indicators for ovarian cancers.[6] Likewise, lysophosphatidylcholine (LPC) levels in plasma has been suggested as potential biomarkers of colorectal cancer.[7] Accordingly, many analytical methods have been proposed for targeted lipid profiling, which included optimal extraction method, ionization additives, chromatographic separation, and ionization mode of mass spectrometry.[1,8-10] Nonetheless, no single method has been reported for simultaneous measurement of S1P, LPAs, and LPCs particularly due to differential ionization capability of each lipid class. For instance, high-throughput analysis has been reported that focused on LPA and S1P using hydrophobic-interaction chromatograph and triple quadrupole linear ion-trap mass spectrometer with negative ionization mode.[9] Another study proposed mass spectrometric analysis of a range of PLs and LPLs, but it applied both positive and negative ionization to detect LPCs.[1] Similarly, a comprehensive profiling on plasma LPS simultaneously quantified S1P and LPAs, but LPCs were analyzed with different extraction method and LC-MS analysis.[10] Thus, the goal of this study is to develop a single analytical platform (e.g.

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extraction method, ionization additive condition, liquid chromatographic separation, and ionization method for mass spectrometry) for simultaneously detecting the three major LPLs, S1P, LPA and its precursor, LPCs.

Experimental

Chemicals and reagents

S1P was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). LPA (C18:1) and chloroform were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). LC grade ammonium formate was purchased from Fluka (Buchs, Switzerland). LC-MS-grade water, acetonitrile, methanol and formic acid were purchased from Fisher Scientific (Loughborough, UK).

Sample preparation

Human blood samples were obtained from 3 healthy controls without any known clinical symptom, which was approved by the Institutional Review Board of the Yonsei University College of Medicine (4-2014-0520). Plasma samples were pooled and triplicates were independently prepared for lipid profiling. The extraction method was modified based on the Folch method,[11] in which 225 μL MeOH was added to 50 μL plasma sample aliquots and vortexed in 1.5 mL Eppendorf tubes for 10 s. Subsequently, 450 μL chloroform ($CHCl_3$) was added and shaking-incubated for 1 hr followed by phase separation with additional 187.5 μL of H_2O. The lower phase was transferred to a new vial and dried to complete dryness.

LC-MS/MS conditions

The dried samples were re-constituted with 50 μL of 70% ACN for lipid profiling. The samples were chromatographically separated with a 150 × 2.1 mm UPLC BEH 1.7-μm C18 column (Waters) controlled by Ultimate-3000 UPLC system (Thermo Scientific). The mobile phase was 10(50)mM ammonium formate, 0.2% formic acid in water (buffer A) and 0.2% formic acid in ACN(buffer B). LC condition was as follows: equilibration in 10% buffer B for 1 min, 10-75% buffer B gradient over 7.5 min, 75-95% buffer B gradient over 7.6 min, 95% buffer B for 2.8 min and re-equilibration in 10% buffer B for 5.5 min. Injection volume of sample was 10 μL for both scan MS profiling (MS1) and MS/MS analysis. MS analyses were performed using the Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific). Mass spectrometer was operated in the full-scan MS/data-dependent MS/MS mode. All raw data (.raw file) were converted to Analysis Base File (ABF) format using Reifycs Abf Converter followed by data process (http://www.reifycs.com/AbfConverter/index.html). Peak detection, deconvolution, peak alignment, and peak annotation were done using the MS-Dial software[12] and LipidBlast libraries.[13] The integration of peak area and manual validation of MS/MS were performed by Tracefinder software (Thermo Scientific, Bremen, Germany).

Results and Discussion

Optimization of LC-MS

LC/MS conditions were optimized for the analysis of S1P and LPA (C18:1). Since S1P are preferentially detected in positive ionization mode, but negative ionization mode is more compatible with LPAs, ionization mode was first evaluated. At the same time, the performance of ionization additive (ammonium formate) was tested with three different concentrations (0, 10, and 50 mM) using reference standard compounds, S1P and LPA (C18:1). In ESI/MS analysis with positive ionization, $[M+H]^+$ ions were analyzed at *m/z* of 380.259 in MS spectrum of S1P at all levels of ammonium formate. The peak areas of the qualifier ion were present at the highest level in 0 mM concentration (Table 1). LPA (C18:1) was measured at *m/z* of 437.2665 in MS spectrum as $[M+H]^+$ ions. The integrated area of the base peak chromatograms (BPCs) was present at higher level in 10 mM concentration of ammonium formate compared to 50 mM condition whereas no qualifier ion was observed in 0 mM condition (Table 1). Under negative ionization, ESI-MS analysis showed *m/z* of 378.2423 as dominant parent ion ($[M-H]^-$) of S1P. Similar to the positive ionization mode, the integrated peak areas were ordered as follows: 0 mM > 10 mM > 50 mM (Table 1). LPA (C18:1) was identified as *m/z* 435.2521 in MS spectrum of $[M-H]^-$ by ESI/MS analysis only in 10 mM and 50 mM of ammonium formate. In summary, ESI-MS with negative ionization mode presented the good performance for the analysis of S1P and LPA in both 10 mM and 50 mM condition; thus, we determined to further evaluate both concentrations of ammonium formate under negative ionization mode using biological sample matrix.

Table 1. Comparisons of integrated area of qualifier ions of S1P and LPA (C18:1) by ionization mode and ammonium formate levels.

The concentration of Ammonium formate	S1P		LPA (C18:1)	
	Positive	Negative	Positive	Negative
0 mM	***	***	X	X
10 mM	**	**	**	***
50 mM	*	*	**	***

[*] is calculated based on peak area
[*] fair [**] good [***] excellent

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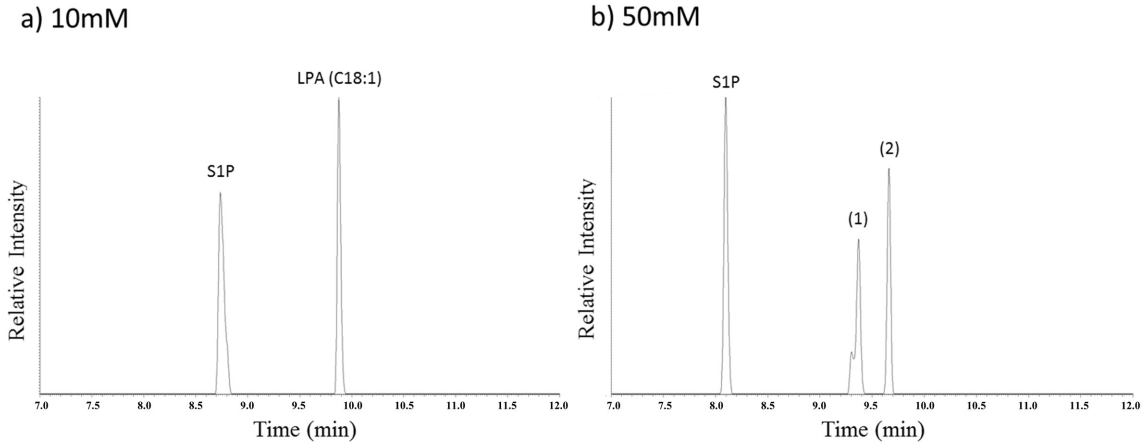

Figure 1. Base peak chromatograms of S1P and LPA with negative ionization mode in a) 10 mM and b) 50 mM concentration of ammonium formate with 0.2% formic acid.

Table 2. Comparisons of analytical precision and integrated area by ammonium formate levels in human blood plasma.

	S1P				LPA (C18:1)			
	Retention time		Peak area of qualifier ion (m/z 378.2423)		Retention time		Peak area of qualifier ion (m/z 435.2521)	
	Average RT	%CV	Relative peak area*	%CV	Average RT	%CV	Relative peak area*	%CV
10mM	8.73	0.06	100	22	9.88	0.06	100	2
50mM	8.13	0.5	80	20	9.68	0.2	27	25

* is calculated based on peak area

Method validation with human sample

The analytical method was further examined to evaluate the applicability to human blood plasma samples with both 10 mM and 50 mM conditions. For determination of intra-assay precision, triplicates of each condition were prepared and analyzed by full-scan MS mode and data-dependent MS/MS. The samples were independently extracted, speed vacuumed complete to dryness, and reconstituted. Figure 1 shows the BPCs of LC-ESI/MS runs obtained. BPCs of S1Ps were uniquely identified in both additive conditions whereas two BPCs were detected as LPA (*m/z* of 435.2521) in 50 mM conditions (Fig. 1). The peak that eluted at 9.88 min (2) was putatively annotated as LPA by tandem mass spectrum but the fragment pattern of the first peak (1) was not matched to library. For analytical precision, percentage of coefficient variation (%CV) was calculated for relative retention times and integrated peak areas of qualifier ions. The higher reproducibility was observed in retention time with 10 mM (%CV of < 0.06%) compared to 50 mM condition (%CV of < 0.5%) (Table 2). Likewise, 10 mM of ammonium formate presented higher analytical precision and the integrated peak areas of the qualifier ions (Table 2). Last, we examined whether the method can be extended to the analysis of other types of LPLs. Accordingly, ms/ms spectra were primarily analyzed using MS-DIAL[12] and annotated against LipidBlast ms/ms spectral libraries.[13] Note that 17 lyso-phosphatidic acids (LPCs) were identified, which has not been reported for the MS analysis in negative ionization method. The CID spectra of the LPCs showed the characteristic ion, *m/z* of 168.04 from the phosphocholine cation. The class of the LPCs covered a various type of acyl chains including LPC (18:1), a precursor of biologically important LPA (C18:1) (Table 3). In addition, 19 LPEs with various types of acyl-chains were annotated based on the fragmentation patterns, and the BPCs were distinctively identified (Fig. 2). The characteristic head group information was acquired from *m/z* of 196.03 for LPE. Figure 2 shows the BPCs of S1P, LPA, LPCs, and LPEs from human blood samples. Detailed information was summarized in Table 3, which included the name, mz of parent ion, characteristic ms/ms fragment (product ion), and retention time.

Conclusions

This study demonstrates the simultaneous detectability of S1P, LPA, LPCs, and LPEs in a single method including extraction procedure, reverse phase liquid chromatography, and single ionization mode (negative ionization). The method proved effective on the mass spectrometric analysis of complex biological matrix, human blood plasma. In addition, the good performance with full-scan MS mode

Table 3. The list of S1P and lysophospholipids identified using data-dependent MS/MS from human blood plasma.

Compound	RT	PPM error	Parent Ion	Daughter Ion	Compound	RT	PPM error	Parent Ion	Daughter Ion
S1P	8.73	2.64	378.2423 m/z	78.95 m/z	LPE (C16:0)	9.64	3.27	452.2792 m/z	196.038 m/z
LPA (C18:1)	8.73	1.38	435.2521 m/z	152.99 m/z	LPE (C16:1)	9.05	2.29	450.2631 m/z	196.038m/z
LPC (C14:0)	8.83	3.82	512.3008 m/z	224.06 m/z	LPE (C17:0)	9.74	1.57	466.2941 m/z	196.038 m/z
LPC (C15:0)	9.3	3.62	526.3164 m/z	224.06 m/z	LPE (C17:1)	9.45	4.48	464.2798 m/z	196.038 m/z
LPC (C17:0)	9.92	0.01	554.3458 m/z	224.06 m/z	LPE (C18:0)	9.52	0.25	480.3089 m/z	196.038 m/z
LPC (C17:1)	9.56	1.37	552.3309 m/z	224.06 m/z	LPE (C18:1)	9.77	0.27	478.2935 m/z	196.038 m/z
LPC (C18:0)	10.23	3.26	568.3633 m/z	224.06 m/z	LPE (C18:2)	9.31	1.22	476.2783 m/z	196.038 m/z
LPC (C18:1)	9.66	3.36	566.3477 m/z	224.06 m/z	LPE (C18:3)	8.82	3.02	474.2635 m/z	196.038 m/z
LPC (C18:2)	9.74	3.82	564.3323 m/z	224.06 m/z	LPE (C20:1)	10.44	3.42	506.3264 m/z	196.038 m/z
LPC (C18:3)	8.35	3.92	562.3167 m/z	224.06 m/z	LPE (C20:2)	9.35	1.03	504.3085 m/z	196.038 m/z
LPC (C18:4)	8.45	0.44	560.2986 m/z	224.06 m/z	LPE (C20:3)	9.55	4.84	502.2958 m/z	196.038 m/z
LPC (C19:1)	10.09	1.82	580.3625 m/z	224.06 m/z	LPE (C20:4)	9.29	1.16	500.2783 m/z	196.038 m/z
LPC (C20:0)	11.32	3.45	596.3948 m/z	224.06 m/z	LPE (C20:5)	8.69	1.06	498.2626 m/z	196.038 m/z
LPC (C20:1)	10.3	4.05	594.3795 m/z	224.06 m/z	LPE (C22:0)	6.39	2.09	536.3705 m/z	196.038 m/z
LPC (C20:2)	9.91	4.14	592.3639 m/z	224.06 m/z	LPE (C22:1)	10.46	2.94	534.3544 m/z	196.038 m/z
LPC (C20:4)	9.31	0.25	588.33 m/z	224.06 m/z	LPE (C22:3)	9.57	0.62	530.325 m/z	196.038 m/z
LPC (C22:4)	9.76	0.23	616.3613 m/z	224.06 m/z	LPE (C22:4)	9.75	0.91	528.3095 m/z	196.038 m/z
LPC (C22:5)	9.43	4.57	614.3486 m/z	224.06 m/z	LPE (C22:5)	9.41	2.53	526.2947 m/z	196.038 m/z
LPC (C22:6)	9.26	2.21	612.3315 m/z	224.06 m/z	LPE (C22:6)	9.14	0.73	524.2781 m/z	196.038 m/z

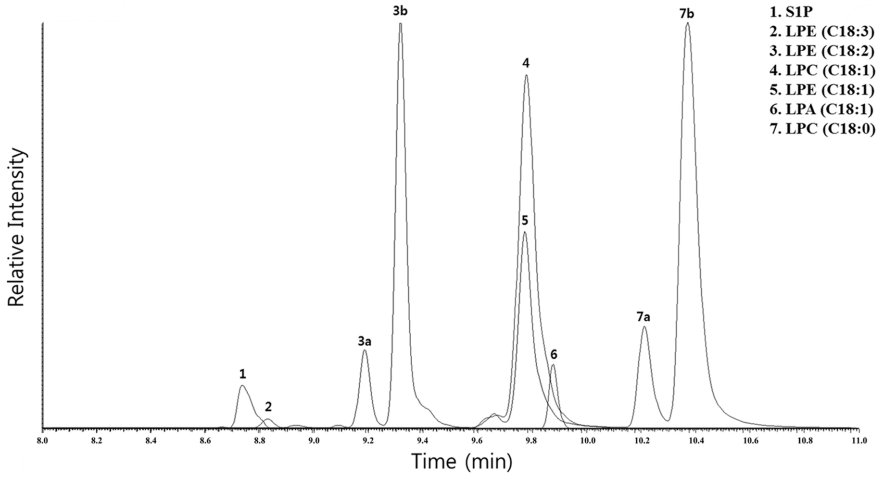

Figure 2. Base peak chromatograms of human plasma from LC-ESI-MS in negative ionization mode.

allows the higher applicability of the procedure, which can be extended to non-targeted profiling of a broad range of lipid species. In addition, reduced amount of salt, ammonium formate (10 mM) may increase the instrumental feasibility.

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