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Development of an MRM based phospholipid profiling method in human plasma using an inert C18 column

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1. Overview

- The wider targeted monitoring method of human plasma phospholipids has been developed as a new tool for biomarker discovery.
- An LC/MS system combined with ultra fast triple quadrupole mass spectrometer LCMS-8060NX, *Nexera*™ UHPLC and a new inert C18 ″Shim-pack Scepter™ Claris” column were used.
- Totally 662 MRM transitions were piled up on 20 minutes chromatographic condition. Over 170 phospholipids were detected in human plasma within CV of 50% without significant carryover.

2. Introduction

Plasma phospholipids are attractive targets to finding disease biomarker or pathophysiological research. Here we developed a phospholipid profiling method in human plasma based on multiple reaction monitoring, MRM, coupled with reversed phase chromatography using inert C18 column. Totally 170 phospholipids were detected including 130 diacyl-phospholipids, 28 lyso-phospholipids and 12 sphingomyelins in 20 minutes’ analytical cycle. Fatty acid composition was determined for all the 130 diacyl-phospholipids by detecting corresponding fatty acid product ion in negative ion mode. Curved gradient was effective to differentiate fatty acid composition isomers such as phosphatidylcolins, PC 18:2_20:4 and 18:1_20:5, and 16:0_22:6. Reproducibility was evaluated by 50 times repeated analyses of human plasma extract.

3. Methods and Materials

Pooled heparin human plasma was purchased from (Kojin-Bio Corp., Japan). An LC-MS system consisting of *Nexera* UHPLC and an LCMS-8060NX mass spectrometer (Shimadzu Corp.) was used. Twenty mM ammonium formate and acetonitrile/isopropanol (1/1, v/v) were used for mobile phase A and B, respectively. Shim-pack Scepter Claris C18, 2.1 x 100 mm, 1.9 µm, (Shimadzu Corp.) was used for chromatographic separation. Polarity switching time was 5 msec. Both dwell time and pause time were set at 1 msec for an MRM transition. One mL of methanol containing 0.1% formic acid was added to 20 µL of human plasma and vigorously mixed a few minutes. After centrifugation, 3 µL of supernatant was injected to LC-MS system.

MS conditions (LCMS-8060NX)

Ionization	ESI, Positive/Negative
Nebulizing Gas Flow	3.0L/min.
Drying Gas Flow	10.0L/min.
Heating Gas Flow	10.0L/min.
DL Temp.	250 ºC
Block Heater Temp.	400 ºC
Interface Temp.	150 ºC
CID Gas Pressure	270 kPa

Ultra Fast Mass Spectrometer
UF Polarity Switch in 5 msec)
UF MRM (Max. 555/sec)

Figure 1 LCMS-8060NX triple quadrupole mass spectrometer

4. Results

4-1. Method development

For phospholipid profiling in human plasma, 662 MRM transitions were set on 20 minutes chromatographic condition using a new inert C18 column. An MRM to monitor polar head group such as colin derived *m/z* 184, and one or two MRM were set for a diacyl-phospholipid with the same fatty acids or different ones, respectively. As shown in Figure 2 for PC 38:6, 11 MRM were set including one for polar head group in positive ion mode and other 10 MRM for monitor fatty acids in negative ion mode. Three isomers PC 18:2_20:4, 18:1_20:5, and 16:0_22:6 were assigned at 10.15 min, 10.25 min, and 10.45 min, respectively. The other isomers PC 16:1_22:5 and 18:3_20:3 were monitored but not detected.

Human Plasma 20 µL
↓
0.1% FA/Methanol 1 mL
↓
3 µL injected on LC/MS
↓
MRM chromatograms

Column	Shim-pack Scepter Claris C18
Injection	3 µL
Flow rate	0.3 mL/min
Elution buffer	A: 20 mM Ammonium formate B: Acetonitrile/Isopropanol (50/50)
Oven Temp.	50˚C
Gradient (GE)	A curved gradient was used.

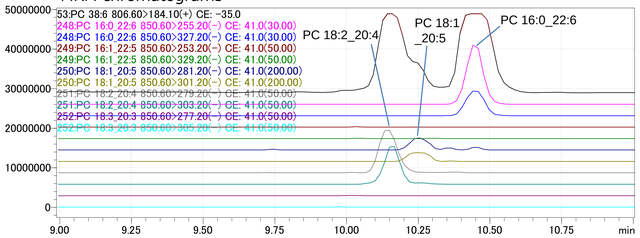

Figure 2 Pre-treatment workflow, chromatographic conditions, and MRM chromatograms for PC 38:6.

4-2. Phospholipids in human plasma

Table 1 170 phospholipids detected in human plasma. CV and mean area values for 50 repeated analyses. Each lipid class was listed in order of area.

	Compound name	Mean area, N=50	CV%		Compound name	Mean area, N=50	CV%
1	LPC 16:0	12590891	5.5	69	PC 18:2_22:1	20967	40.1
2	LPC 18:0	10586633	6.5	70	PC 18:2_22:0	20693	28.3
3	LPC 18:2	5366848	6.8	71	PC 14:0_18:0	20314	38.9
4	LPC 18:1	4262403	7.3	72	PC 14:0_16:1	18351	42.5
5	LPC 20:4	599730	10.2	73	PC 14:0_14:0	16715	41.3
6	LPC 16:1	446275	12.4	74	PC 20:2_20:3	13224	48.8
7	LPC 17:0	371317	8.7	75	PC 20:1_20:3	12652	44.0
8	LPC 14:0	317125	8.2	76	PC 14:0_22:6	12605	46.1
9	LPC 20:3	314656	14.0	77	PC 18:3_20:4	11102	36.5
10	LPC 18:3	90463	11.9	78	PC 16:1_18:3	9065	41.0
11	LPC 20:2	89312	14.0	79	PC 20:0_20:3	8859	49.0
12	LPC 20:1	89018	13.2	80	PC 14:0_18:3	8356	42.2
13	LPC 15:0	86394	8.4				
14	LPC 17:1	55291	12.5	1	LPE 18:0	1535415	13.0
15	LPC 20:0	50618	15.1	2	LPE 18:2	1274312	12.6
16	LPC 22:5	22311	22.3	3	LPE 18:1	631482	8.5
17	LPC 20:5	19539	15.3	4	LPE 16:0	468721	8.7
18	LPC 22:6	15240	20.6	5	LPE 20:4	261270	12.1
19	LPC 22:4	12977	20.0	6	LPE 20:3	27132	18.7
				7	LPE 16:1	14670	18.9
1	PC 16:0_18:2	58965589	5.7	8	LPE 22:6	14210	19.9
2	PC 18:0_18:2	46587815	6.5				
3	PC 16:0_18:1	30741183	7.0	1	PE 18:0_20:4	1568647	8.7
4	PC 16:0_20:4	12579677	6.2	2	PE 18:0_18:2	1428584	10.5
5	PC 18:2_18:2	7641465	9.8	3	PE 18:1_18:1	568689	17.5
6	PC 18:0_20:4	6600442	8.0	4	PE 18:0_22:6	432873	12.3
7	PC 18:0_20:3	5237183	9.8	5	PE 16:0_18:2	408937	11.0
8	PC 16:0_20:3	4653924	8.3	6	PE 16:0_22:6	384514	17.6
9	PC 18:1_18:1	4268667	8.2	7	PE 18:1_18:2	374369	12.5
10	PC 18:0_18:1	4050703	9.2	8	PE 16:0_20:4	368134	11.0
11	PC 18:1_18:2	4005264	7.4	9	PE 18:0_18:1	339326	14.3
12	PC 16:0_16:0	2408516	9.7	10	PE 18:2_18:2	289988	15.7
13	PC 18:0_22:6	1989134	10.3	11	PE 18:1_20:4	159165	18.2
14	PC 16:0_22:6	1877485	9.0	12	PE 16:0_18:1	138010	22.2
15	PC 16:1_18:3	1568830	8.6	13	PE 18:0_20:3	121059	23.3
16	PC 16:1_18:2	1441655	10.9	14	PE 18:0_22:5	85505	25.9
17	PC 16:0_16:1	1378650	10.1	15	PE 18:2_20:4	68122	23.7
18	PC 16:0_20:2	1182801	10.1	16	PE 16:0_22:5	67460	16.3
19	PC 18:1_20:4	1140317	10.1	17	PE 16:0_20:3	66800	30.9
20	PC 18:1_20:3	1038014	11.9	18	PE 18:0_20:5	43813	31.2
21	PC 16:0_22:4	960176	11.9	19	PE 16:0_22:4	32222	8.2
22	PC 18:2_20:4	890457	10.6	20	PE 18:0_22:4	30734	44.5
23	PC 16:1_18:1	859248	12.4	21	PE 18:0_18:3	23283	44.4
24	PC 18:0_22:4	722544	12.1	22	PE 16:0_18:0	14284	20.0
25	PC 16:0_22:5	698004	9.3	23	PE 16:0_16:1	10187	39.8
26	PC 16:0_20:5	588343	12.5				
27	PC 16:0_18:0	559245	11.8	1	LPI 18:2	119082	11.2
28	PC 16:0_20:2	1182801	10.1				
29	PC 18:1_20:4	1140317	10.1	1	PI 18:0_20:4	8877499	9.3
30	PC 18:1_20:3	1038014	11.9	2	PI 18:0_18:2	4634368	10.9
31	PC 16:0_22:4	960176	11.9	3	PI 18:0_18:1	1139079	11.9
32	PC 18:2_20:4	890457	10.6	4	PI 16:0_18:2	1137158	11.1
33	PC 18:1_18:1	859248	12.4	5	PI 18:0_20:3	1132277	17.0
34	PC 18:0_22:4	722544	12.1	6	PI 16:0_20:4	850955	13.3
35	PC 16:0_22:5	698004	9.3	7	PI 18:1_18:2	681031	16.8
36	PC 16:0_20:5	588343	12.5	8	PI 18:1_18:1	657390	12.5
37	PC 16:0_18:0	559245	11.8	9	PI 16:0_18:1	558950	12.1
38	PC 18:0_18:0	523205	15.4	10	PI 18:0_22:6	350873	15.6
39	PC 18:0_20:5	487668	12.8	11	PI 18:1_20:4	289337	33.9
40	PC 18:2_20:2	387673	17.4	12	PI 18:0_22:5	176527	17.8
41	PC 17:0_18:2	362518	13.4	13	PI 16:0_20:3	150091	20.1
42	PC 18:0_22:5	362417	17.9	14	PI 16:0_16:1	128075	18.7
43	PC 14:0_18:1	348127	13.1	15	PI 16:0_16:0	127246	19.5
44	PC 18:0_20:2	315901	14.2	16	PI 16:1_18:0	121160	19.8
45	PC 18:0_18:3	314397	17.1	17	PI 16:0_22:6	100834	19.7
46	PC 18:2_20:1	274734	15.2	18	PI 18:0_22:4	92649	23.8
47	PC 18:2_20:3	262792	15.1	19	PI 16:0_22:5	87496	17.1
48	PC 16:0_20:1	251451	14.6	20	PI 18:0_20:2	85624	21.8
49	PC 18:2_20:0	249253	15.3	21	PI 18:2_18:2	60805	21.9
50	PC 14:0_18:2	246595	14.4	22	PI 18:0_20:5	43190	37.5
51	PC 18:1_18:3	206099	17.8	23	PI 18:0_18:3	43105	39.3
52	PC 16:1_18:0	188327	17.0	24	PI 16:0_22:4	35770	36.6
53	PC 14:0_16:0	166944	16.0	25	PI 18:1_20:3	31629	40.0
54	PC 18:1_22:6	153107	21.6	26	PI 16:1_18:1	28354	34.0
55	PC 18:2_18:3	147099	21.7	27	PI 16:0_20:2	25429	49.3
56	PC 18:1_20:2	133449	25.1				
57	PC 17:0_20:4	132322	26.5	1	SM 18:1;O2/16:0	17142983	5.9
58	PC 17:0_18:1	126394	18.2	2	SM 18:1;O2/22:0	6624463	5.8
59	PC 18:1_20:1	106560	22.8	3	SM 18:1;O2/18:0	3366477	6.8
60	PC 16:1_20:3	105760	28.1	4	SM 18:1;O2/22:1	3293764	7.5
61	PC 16:1_20:4	103850	29.6	5	SM 18:1;O2/20:0	3213034	6.7
62	PC 15:0_18:2	96415	22.3	6	SM 18:1;O2/16:1	3201541	7.5
63	PC 20:4_20:4	81609	22.8	7	SM 18:1;O2/18:1	1921720	7.3
64	PC 18:2_22:6	66777	22.5	8	SM 18:1;O2/14:0	1410925	7.3
65	PC 14:0_20:4	66737	22.6	9	SM 18:1;O2/20:1	899574	10.8
66	PC 18:1_20:5	64989	25.1	10	SM 18:1;O2/18:2	119539	21.3
67	PC 20:0_20:4	60219	33.9	11	SM 18:1;O2/20:2	30145	40.3
68	PC 18:1_22:4	59651	30.7	12	SM 18:1;O2/18:3	10910	44.0

- Phosphatidyl colins, LPC and PC were more than half in the 170 phospholipids as shown in Figure 3.
- Reproducibility was estimated by 50 times repeated analyses of human plasma. 107 of 170 phospholipids were within 20% CV (Figure 4).
- In the chromatograms for 22:5 including phospholipids, probable structural isomers were detected as arrowed peaks in Figure 5 and shown in red on Table 1.

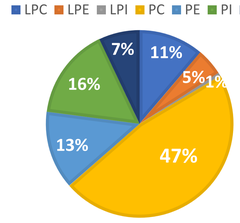

Figure 3 The percentage of 170 phospholipids were shown.

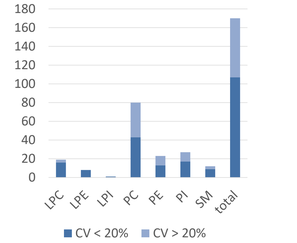

Figure 4 The number of phospholipids less or more than 20% of CV.

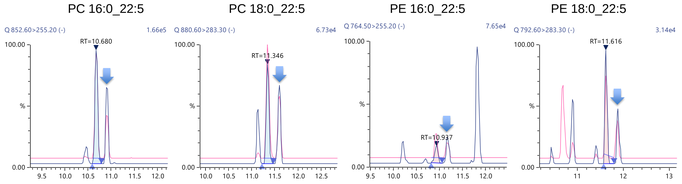

Figure 5 Probable structural isomers of fatty acid 22:5 including phospholipids were arrowed on MRM chromatograms for PC 16:0_22:5, PC 18:0_22:5, PE 16:0_22:5, and PE 18:0_22:5. The assigned peak was shown with retention time.

5. Conclusions

- We have developed a phospholipid profiling method with 20 minutes chromatographic condition. Totally 170 phospholipids were detected. Fatty acid combination was assigned for all 130 diacyl-phospholipids.
- Carryover could be estimated below 0.01% by most abundant peak area of PC 16:0_18:2 in methanol blank analysis after plasma analysis (data not shown).
- Omega-3 fatty acids such as EPA 20:5 and DHA 22:6 bound to phospholipids were clearly detected as shown in blue on Table 1.
- We believe that this method is available for evaluation of “fatty acid balance” in human plasma sample.

Acknowledgement

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