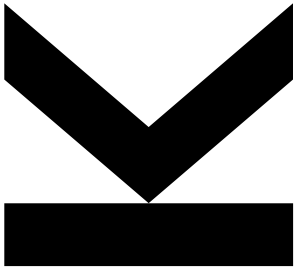
UPDATE: ANALYSIS OF PHOSPHATIDYLCHOLINE SPECIES IN CLINICAL SAMPLES



Bernd Reichl
19. Oct 2017

JOHANNES KEPLER
UNIVERSITÄT LINZ

Interreg
EUROPÄISCHE UNION
Österreich-Tschechische Republik
Europäischer Fonds für regionale Entwicklung

Overview

1st batch of samples

- 25 samples (16 CSF + 9 serum)
- Total of 8 test persons
- 2-4 samples per test person (either 2 CSF or 2 CSF + 2 serum)
- 12 directly comparable pairs of samples (sample 24 has no comparable partner sample)

Analytical approach

- Lipid extraction from 100 µL sample aliquot
- Chromatographic separation (Agilent 1260 series HPLC, RP C18 column)
- Targeted MS detection of 41 PC/LPC species (LTQ Orbitrap XL mass spectrometer)
- Relative quantification

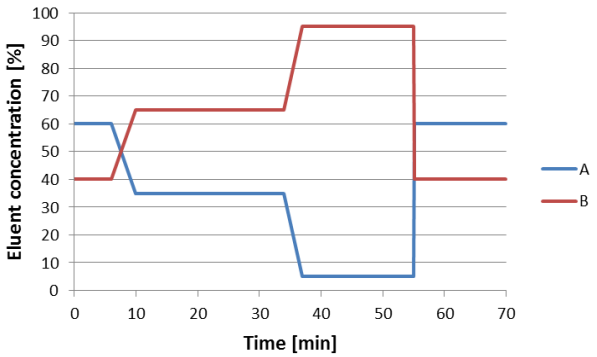
Experimental

Extraction

- Several methods published: Folch (1957), Bligh & Dyer (1959), MTBE, BUME,...
- Our approach: acidified Bligh & Dyer
  - Liquid liquid extraction method ($CHCl_3$, MeOH, 10 mM HCl)
  - 3 extraction steps
  - $CHCl_3$ phases are collected and brought to dryness with N2 stream
  - Redissolution in HPLC eluent
- Recovery (tested adding 5 Standards) > 80%

Chromatographic separation

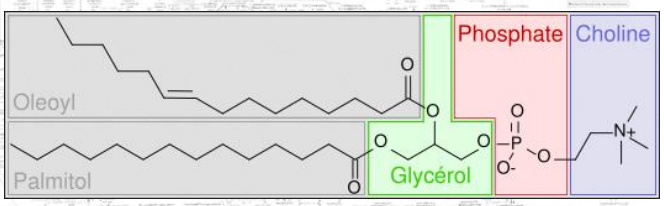
- Phenomenex Kinetex C18 column (150 x 3 mm, 2,6 µm)
- Separation protocol based on Uhl et al 2011:
  - Eluent A (60/40 H2O/MeOH + 10 mM NH4-Ac + 1 mM HAc)
  - Eluent B (90/10 IPA/MeOH + 10 mM NH4-Ac + 1 mM HAc)
- Flowrate: 0,25 ml/min Injection volume: 10 µL



Experimental

Mass spectrometry – LTQ Orbitrap XL

- ESI positive mode
- Data dependent MS^2
  - 41 target analytes (8 LPC + 33 PC species)
  - Full scan in Orbitrap → exact mass
  - MS^2 fragments scan in linear ion trap → characteristic fragment



} Species characterization (e.g. PC aa C32:1)

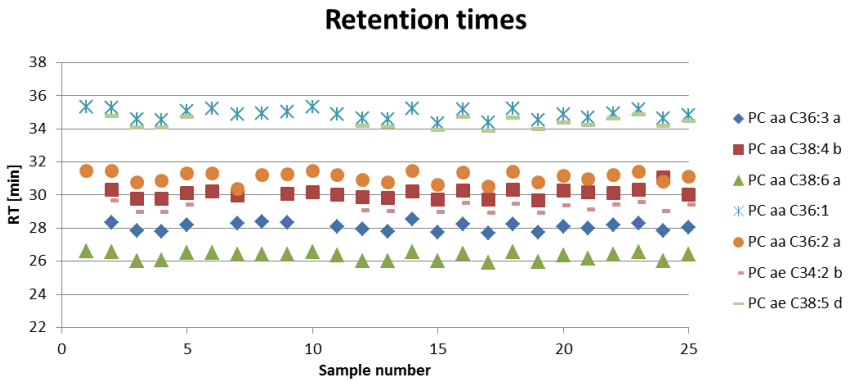
Data analysis

- TraceFinder Software (Thermo)
- Quantification:
  - External calibration with 5 PC/LPC standards
  - 2 calibration ranges: CSF samples 10 ppb – 2 ppm; Serum samples 5 ppb – 40 ppm
  - Internal standard (added before extraction): PC aa C17:0 C17:0
  - Peak areas > 200.000 a.u. are quantified (~ 5 ppb)
  - Relative quantification using structurally most similar standard

JKU

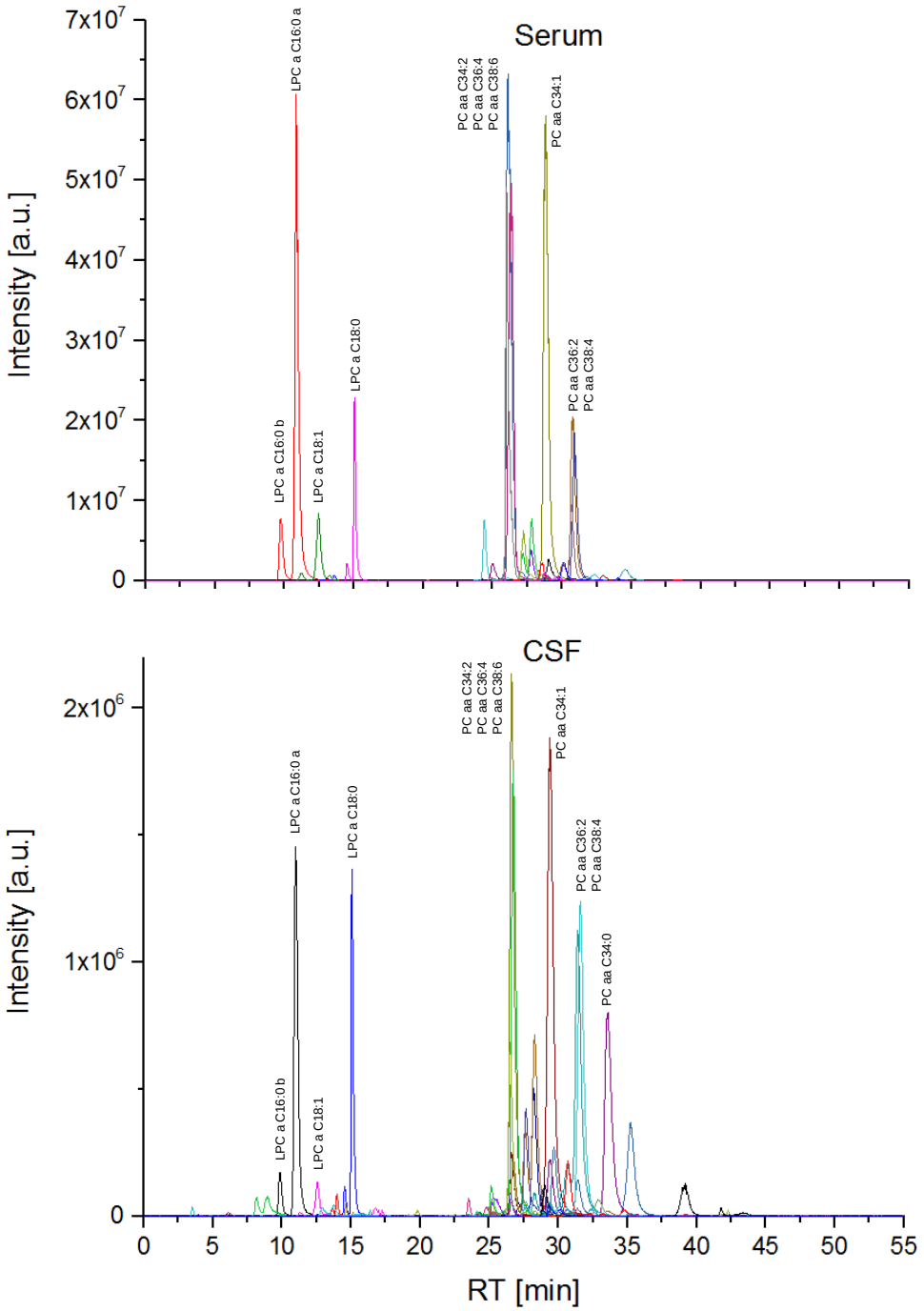
Experimental

- 2 sequences run (each 25 samples + standards + controls)
  - ~ 60 injections
  - > 3 days runtime
  - 1st sequence: serum samples out of calibration range, but CSF samples OK
  - 2nd sequence: serum samples
- Linearity CSF: 10 ppb – 2 ppm ($R^2 > 0,97$)
- Linearity Serum: 5 ppb – 40 ppm ($R^2 > 0,96$)
- Range of analyte concentrations:
  - CSF: 10 ppb – 6 ppm
  - Serum: 60 ppb – 160 ppm
- Mass accuracy: < 3,6 ppm (no lock mass used yet)
- RT´s: low variance over whole sequence
  - RSD´s ≤ 1%
  - Max. deviation: average + 3%
  - Column is thoroughly flushed after ~10 injections



Chromatograms

- Overlay of EIC´s for all targets
- Serum vs. CSF (pooled control samples)
- Clear separation of PCs and LPCs
- Partially coelution of species



Test person	G	G		G	G	
Sample Type	S	S	% Change	L	L	% Change
Date	01.12.2014	18.06.2015		04.12.2014	19.06.2015	
Sample number	Sample 19	Sample 21		Sample 20	Sample 22	
LPC a C18:0 a	8359	15703	88%	343		
LPC a C18:0 b	1252	2480	98%	26		
LPC a C18:1 a	6561	19201	193%	378		
LPC a C18:1 b	797	1990	150%	19		
LPC a C18:1 c	774	2564	231%	42		
PC aa C30:0	228	1147	404%	32	16	-51%
PC aa C32:0	4205	4881	16%	357	139	-61%
PC aa C32:1	3350	18751	460%	666	53	-92%
PC aa C34:1	43493	113619	161%	6636	737	-89%
PC aa C34:2	63136	99533	58%	3975	119	-97%
PC aa C36:1	2838	19657	593%	1857	110	-94%
PC aa C36:2 a	17257	46144	167%	2396	76	-97%
PC aa C36:2 b	3631	21807	501%	1192	108	-91%
PC aa C36:3 a	10100	25903	156%	990	14	-99%
PC aa C36:3 b	3980	20281	410%	849	13	-98%
PC aa C36:4	33473	26483	-21%	1453	91	-94%
PC aa C36:5	4136	8591	108%	217		
PC aa C38:4 a	10538	10437	-1%	842	80	-91%
PC aa C38:4 b	474	1294	173%	150	8	-94%
PC aa C38:5 a	6472	9467	46%	453	20	-96%
PC aa C38:5 b	1707	4175	145%	205		
PC aa C38:6 a	25550	17666	-31%	941	29	-97%
PC aa C38:6 b	423	2459	481%	93		
PC aa C40:6	4993	5102	2%	405	18	-95%
PC ae C32:1 a	207	280	35%	35		
PC ae C32:1 b	118	163	38%	9		
PC ae C34:1	908	1256	38%	112	18	-84%
PC ae C34:2 a	696	1153	66%	77	16	-80%
PC ae C34:2 b	321	364	13%	30		
PC ae C36:4 a	2129	1772	-17%	109		
PC ae C36:4 b				26		
PC ae C36:5 a	2129	2147	1%	119	8	-94%
PC ae C36:5 b	104	283	171%	9		
PC ae C38:5 a	2769	2404	-13%	130		
PC ae C38:5 c	404	308	-24%	12		
PC ae C38:5 d				30		
PC ae C38:6 a	470	310	-34%	40		
PC ae C38:6 b	966	863	-11%	14		
PC aa C34:0	1889	1890	0%	1041	1041	0%

Test person	A	A	
Sample Type	L	L	% Change
Date	21.07.2014	05.12.2014	
Sample number	Sample 01	Sample 02	
LPC a C16:0 a		55	
LPC a C16:0 b		5	
LPC a C17:0			
LPC a C18:0 a		12	
LPC a C18:0 b			
LPC a C18:1 a		12	
LPC a C18:1 b			
LPC a C18:1 c			
PC aa C30:0		26	
PC aa C32:0	24	340	1325%
PC aa C32:1	14	130	801%
PC aa C34:1	136	1339	881%
PC aa C34:2	14	276	1895%
PC aa C36:1	18	189	947%
PC aa C36:2 a		139	
PC aa C36:2 b		133	
PC aa C36:3 a		94	
PC aa C36:3 b		43	
PC aa C36:4		228	
PC aa C36:5			
PC aa C38:4 a		196	
PC aa C38:4 b		21	
PC aa C38:5 a		56	
PC aa C38:5 b		13	
PC aa C38:6 a		38	
PC aa C38:6 b			
PC aa C40:6		23	
PC ae C32:1 a		17	
PC ae C32:1 b			
PC ae C34:1		37	
PC ae C34:2 a		17	
PC ae C34:2 b			
PC ae C36:4 a		29	
PC ae C36:4 b			
PC ae C36:5 a		41	
PC ae C36:5 b			
PC ae C38:5 a		34	
PC ae C38:5 c			
PC ae C38:5 d			
PC ae C38:6 a			
PC ae C38:6 b			
PC aa C34:0	1028	1028	0%

Results

- At first sight no clear or common pattern for all sample pairs
- Serum has substantially higher concentrations of PC/LPC species and also contains more different species than CSF
- Concentration changes in CSF samples larger than in serum samples
- Concentrations change in both directions
- 5 highest concentrated PC/LPC species:

  - ranking is very similar in individual samples

  - PC aa C34:1 and PC aa C32:0 have same RT´s as corresp. standards → supposedly PC (16:0/18:1) and PC (16:0/16:0)

  - PC aa C36:2 has different RT as corresponding standard

  → Supposedly different species than PC (18:1/18:1)

	Serum	CSF
1	PC aa C34:2	PC aa C34:1
2	PC aa C34:1	PC aa C34:2
3	PC aa C36:2	PC aa C32:0
4	PC aa C36:4	PC aa C36:2
5	LPC a C16:0	PC aa C36:4

Problems/Questions/Challenges

- Control samples in 2nd sequence: peak areas of all target analytes (including IS) decreased substantially during triplicate analysis (up to - 66%)
  - in 1st sequence this was not the case
  - also IS in samples did not show any trend during sequence.
- Variance in extraction recovery (RSD´s of IS peak areas ~ 20%)
  - CSF: avoid liquid liquid extraction and just do protein precipitation?
  - Serum: deeper evaluation of alternative extraction methods
- Identify lock mass to improve mass acuracy
- TraceFinder: get more skilled
- Further analytes?

JKU

Interreg
EUROPÄISCHE UNION
Österreich-Tschechische Republik
Europäischer Fonds für regionale Entwicklung