Implementation of lipidomics in the clinical routine: can fluoride/citrate blood sampling tubes improve the preanalytical stability?

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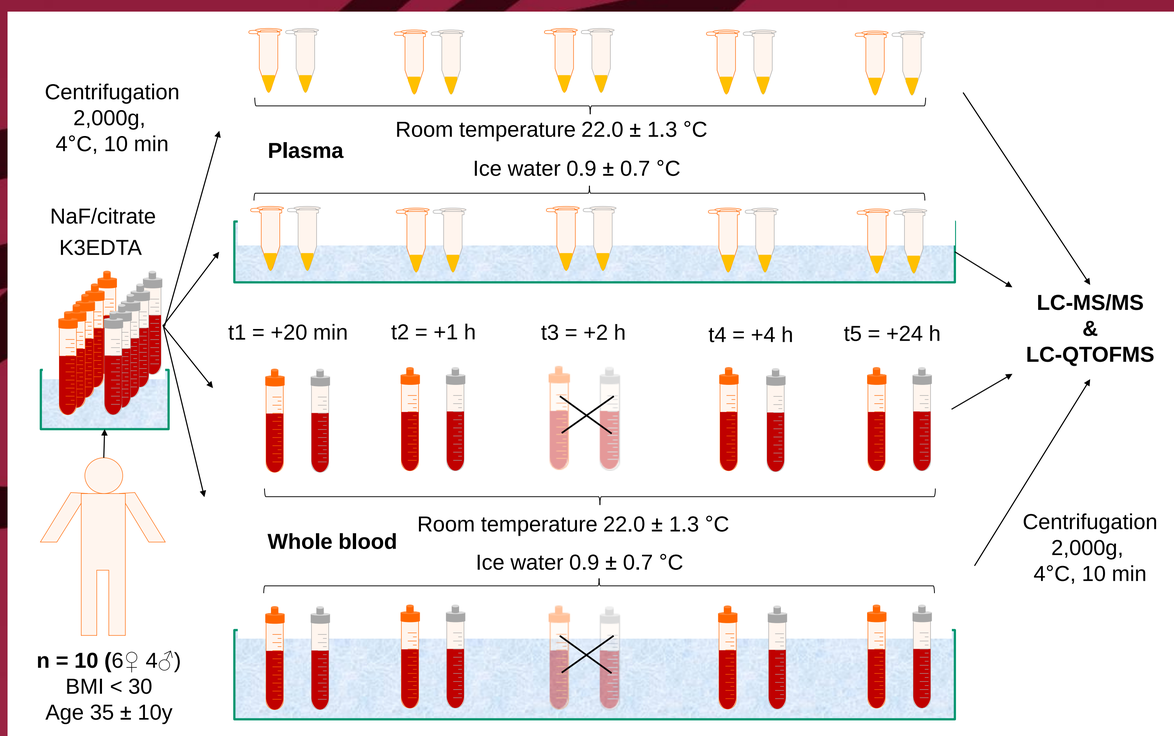
Introduction

Lipid compounds are of great interest as potential biomarkers for several diseases. Discovering biomarkers is very challenging since their suitability must be validated, requiring not only robust analytical procedures but also ensuring the quality of the whole analytical process from sampling to data analysis. In particular, the impact of preanalytical sample handling on analyte stability is a rarely described but very important parameter in analyzing any endogenous compound.

Especially endocannabinoids and lysophosphatidic acids (LPA) were reported to show rapid *ex-vivo* formation at room temperature [1,2]. In the case of endocannabinoids and related compounds, storage of whole blood samples on ice directly after sampling is still insufficient for stabilization [3]. Negligence of the preanalytical stability of LPA might have even led to their false reporting as potential biomarkers for ovarian cancer [4].

Therefore, the primary objective of the study was assessing the preanalytical stability of several lipid mediators in human whole blood and plasma samples under different sampling and storage conditions. Furthermore, commercially available sodium fluoride/citrate tubes (Sarstedt) were tested as a way to improve lipid stability, which could be easily implemented into the clinical routine.

Design of Experiment: Stability Study



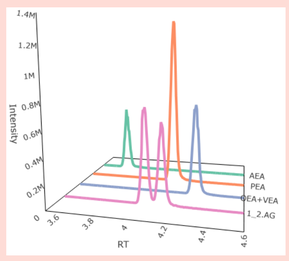
K3EDTA:

- Standard additive for lipid analysis
- Inhibits enzymes with divalent metal ions (Ca^{2+}, Mg^{2+})
- Stability problems for endocannabinoids and LPA [1,2]

Sodium fluoride/citrate:

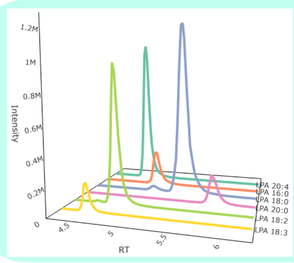
- Fluoride inhibits a broad range of enzymes, including phosphate binding sites [5]
- Citrate binds Ca^{2+}, preventing coagulation
- Acidification prevents isomerization of 2-AG to the biological inactive 1-AG [6]

LC-MS Methodology



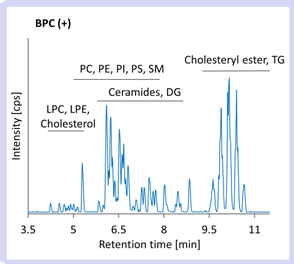
Endocannabinoids:

- Liquid-Liquid Extraction with ethylacetate and hexane
- 200 µL sample volume
- Agilent 1290 Infinity I UHPLC system
- Acquity UPLC BEH C18 column (100 x 2.1 mm, 1.7 µm)
- MRM on a Sciex QTrap 6500+, operated in positive ESI
- 1-/2-arachidonoyl glycerol (AG), anandamide (AEA), oleoyl-ethanolamine/vaccenic acid ethanolamine (OEA/VEA)), palmitoyl-ethanolamine (PEA)



Lysophosphatidic acids (LPA):

- Liquid-Liquid Extraction with n-butanol and Na_2HPO_4/citrate buffer
- 100 µL sample volume
- Agilent 1200 HPLC system
- Luna C18 (2) column (50 x 2 mm, 5 µm)
- MRM on a Sciex QTrap 5500, operated in negative ESI
- LPA 16:0, 18:0, 18:1, 18:2, 18:3, 20:4



Non-targeted lipidomics:

- Liquid-Liquid Extraction with MTBE with 20 µL sample volume
- Shimdazu Nexera-X2 UHPLC system
- Zorbax Eclipse Plus RRHD column (50 x 2.1, 1.8 µm)
- Screening from 100 – 1000 *m/z* (± 5 ppm mass error) & data-dependent acquistion on a Sciex QTOF 6600, operated in negative and positive ESI

References

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[4] T. Yagi, M. Shoaib, C. Kuschner, M. Nishikimi, L.B. Becker, A.T. Lee, J. Kim, Challenges and Inconsistencies in Using Lysophosphatidic Acid as a Biomarker for Ovarian Cancer, Cancers (Basel) 11(4) (2019).
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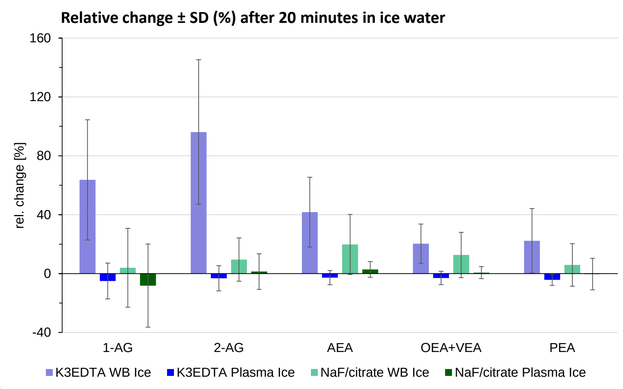
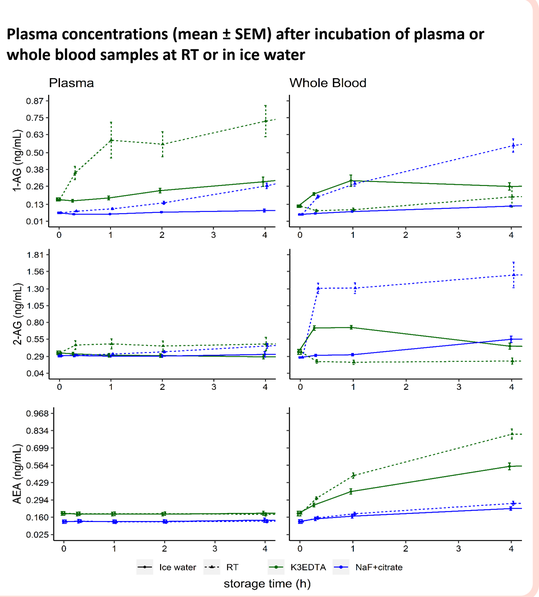
Results

Endocannabinoids:

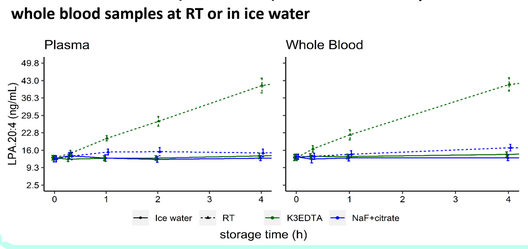
- Increased in K3EDTA whole blood in ice water
- Stability can be improved using NaF/citrate as additive
- Samples should be kept on wet ice

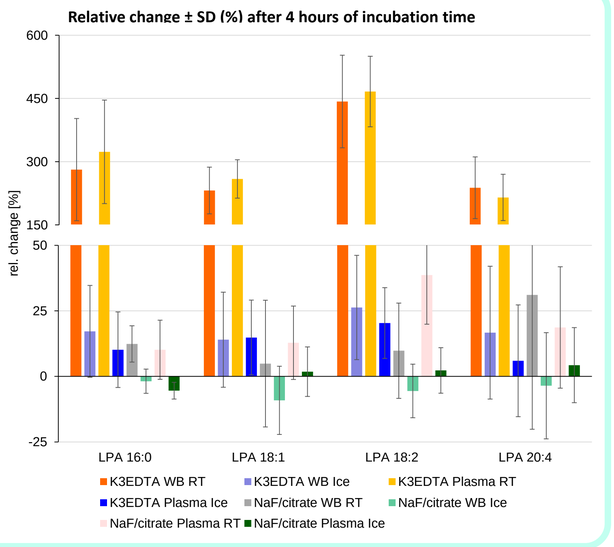




Lysophosphatidic acids:

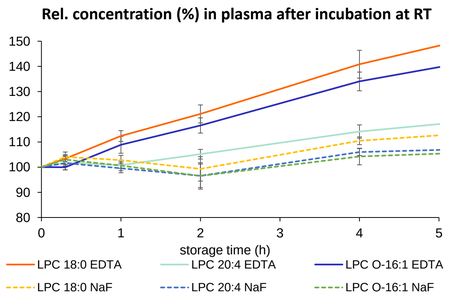
- Rapid *ex-vivo* formation both in whole blood and plasma at room temperature
- Stable for up to 4 hours in ice water
- NaF/citrate greatly improves LPA stability
- NaF/citrate blood/plasma stable at RT for up to 2 hours





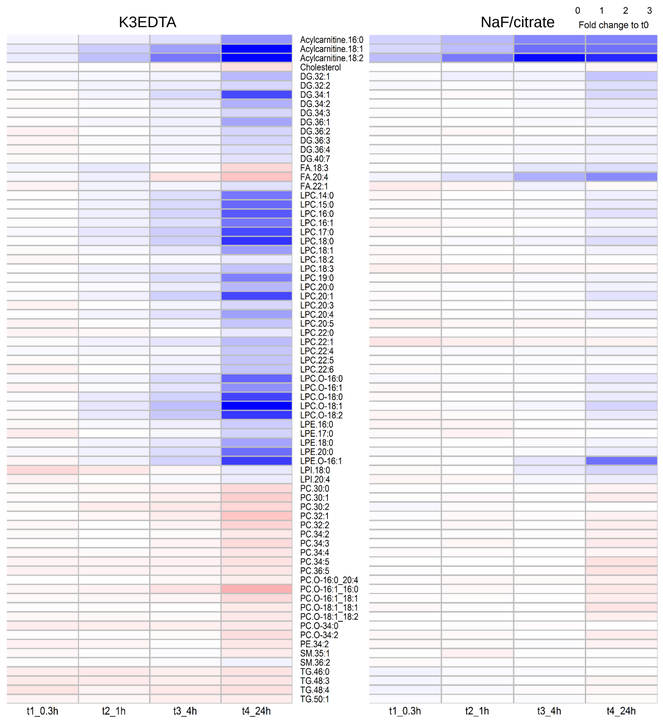
Non-targeted lipidomics:

- Time-dependent increase of free fatty acids, diglycerides, LPC and LPE in plasma (>20% after 4 h) at RT
- Plasma processing at RT is acceptable for < 1 h
- Similar results for whole blood samples
- Whole blood samples should be kept in ice water
- NaF/citrate improves phospholipid stability both in whole blood and in plasma



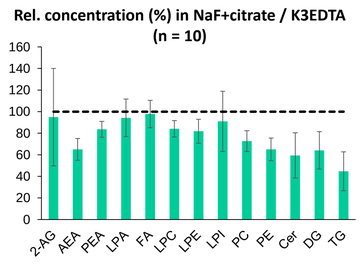
Fold changes after incubation of whole blood at RT

73 unequivocally identified lipids out of the 360 changed features in K3EDTA whole blood are shown, with a FDR < 0.1 (paired t-test) between 24 h of incubation time and control samples (a total of 3198 aligned features was processed, n = 10).



Different baseline levels in K3EDTA and NaF/citrate plasma

- Lowered baseline levels of many lipids were observed in NaF/citrate plasma
- Lipid classes were affected differently (very lipophilic compounds decreased more)
- Total protein conc. in NaF/citrate plasma was decreased by 11 %
- Decreased lipoprotein solubility in NaF/citrate?



Conclusion

Preanalytical stability has to be considered before lipid biomarker searching!

- Endocannabinoid levels in K3EDTA blood were increased after 20 min in ice water
- Increased LPA levels in K3EDTA blood & plasma stored for 20 min at RT
- K3EDTA blood and plasma for lipidomics analysis can be stored for up to 4 h in ice water
- Chilled NaF/citrate blood can be stored for 1 h for endocannabinoid measurement
- NaF/citrate greatly improves phospholipid and endocannabinoid stability
- Baseline levels of several lipid (-mediators) differ between K3EDTA and NaF/citrate

We propose sodium fluoride/citrate as anticoagulant for endocannabinoid measurement!