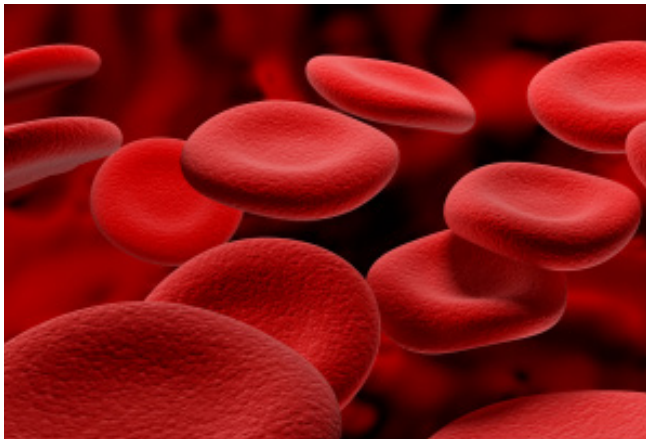
Investigations on storage-induced changes of the red blood cell lipidome

Application note

Doping Control



Authors

Katja Walpurgis 1)*, Thomas Piper 1)*, Folker Wenzel 2), Wilhelm Schänzer 1) & Mario Thevis 1)

1) Center for Preventive Doping Research/Institute of Biochemistry, German Sport University Cologne, Germany;

2) Faculty of Medicine and Life Sciences, Hochschule Furtwangen University, Furtwangen, Germany

Introduction

Lipid biomarkers for RBC storage lesions could be potentially used to develop direct detection strategies for autologous blood doping. Within this study, two complementary approaches for the analysis of the RBC lipidome were developed by using high performance liquid chromatography/time-of-flight mass spectrometry (HPLC/TOF-MS) and gas chromatography/time-of-flight mass spectrometry (GC/TOF-MS). The applicability of both approaches was demonstrated by analyzing an exemplary set of four blood samples which were stored *ex vivo* for a period of 42 days.

Materials and Methods

Blood donations were conducted to collect whole blood samples from healthy volunteers that met the international criteria for blood donation (figure 1) . RBC concentrates were prepared and stored according to standard blood bank conditions for the maximum length of 42 days [1]. For lipid extraction [2], a total of 800 µL of cold chloroform/methanol (2:1, v/v) were added to 200 µL of RBC lysate and the mixture was subjected to ultrasonication (10 min). Following centrifugation (5 min, 16000 x g), the lower lipophilic phase was removed and dried under reduced pressure (30 °C).

Approach 1

High Performance liquid chromatography/electrospray ionization time-of-flight mass spectrometry

For LC-TOF analysis, the dried lipid extracts were solved in acetonitrile/methanol (50/50, v/v). High resolution/high accuracy ESI-MS was conducted using an Agilent (Waldbronn, Germany) 6550 iFunnel Q-TOF LC-MS instrument equipped with a Dual AJS electrospray ion source operated at 25 °C and 3500 V. The mass spectrometer was calibrated using the manufacturers' protocol allowing for mass accuracies < 3 ppm for the period of analysis. The fragmentor voltage was set to 365 V. Separation of lipids was achieved using an Agilent 1290 Infinity LC equipped with an Agilent Eclipse Plus C18 RRHD column (1.8 µm, 2.1 x 50 mm). The solvents were A = methanol/water (50/50, v/v) and B = methanol/acetonitrile (50/50, v/v). The gradient started with 70% A, then decreased to 0% A in 45 min, held for 15 min with subsequent re-equilibration for 5 min. The flow was constant at 0.3 mL/min and the injection volume was 5 µL.

Approach 2

Thin-layer chromatography (TLC and gas chromatography/eletron ionization time-of-flight mass spectrometry

Prior to GC-TOF analysis, the dried lipid extracts were reconstituted in 100 µL of chloroform and separated by means of TLC using concentrating zone plates (Merck, Darmstadt, Germany) and a solvent mixture of chloroform/ethanol/triethylamine/water (30/35/35/7, v/v). The assignment of the separated spots to the different lipid classes was achieved by comparison to corresponding lipid standards (Sigma Aldrich, Steinheim, Germany). In order to analyze the fatty acid composition of the lipids, they were extracted from the scraped silica gel with 600 µL chloroform/methanol (2/1, v/v; 10 min at 1000 rpm followed by 10 min ultrasonication), saponified (1 M KOH/MeOH; 2 h 40 °C), extracted with N-hexane and finally derivatized to trimethylsilyl derivatives (80 µL ethyl acetate, 20 µL MSTFA, 1 h 60 °C) before analysis.

High resolution/high accuracy mass spectrometry following electron ionization was conducted on an Agilent 7890A GC interfaced to a 7200 Accurate-Mass Q-TOF analyzer. The GC was equipped with an Agilent HP-ULTRA 1 column (17 m, inner diameter 0.2 mm, film thickness 0.11 µm), and instrument temperatures were 280 °C for transfer line and injector and 230 °C for the ion source. Helium (purity grade 4.6) was used as carrier gas; injection was conducted in pulsed split mode (5 µL) with a split ratio of 5:1. The GC temperature was programmed from 60 °C (1.5 min) to 120 °C at 40 °C/min, then at 10 °C/min to 325 °C, where the temperature was maintained for another 2 min. MS data were acquired in full scan mode at 2 GHz from m/z 50 to 800.

All data was statistically evaluated using Mass Profiler Professional (Agilent) and Principal Component Analysis (PCA).

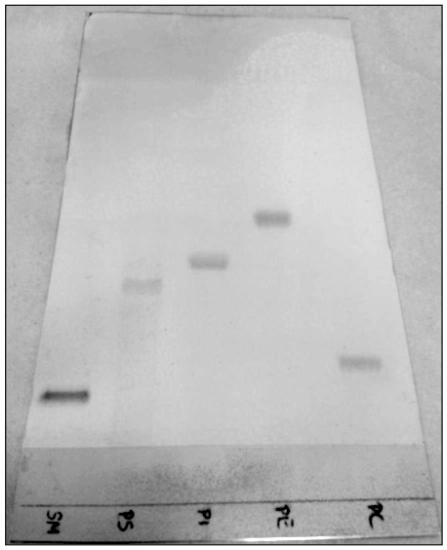
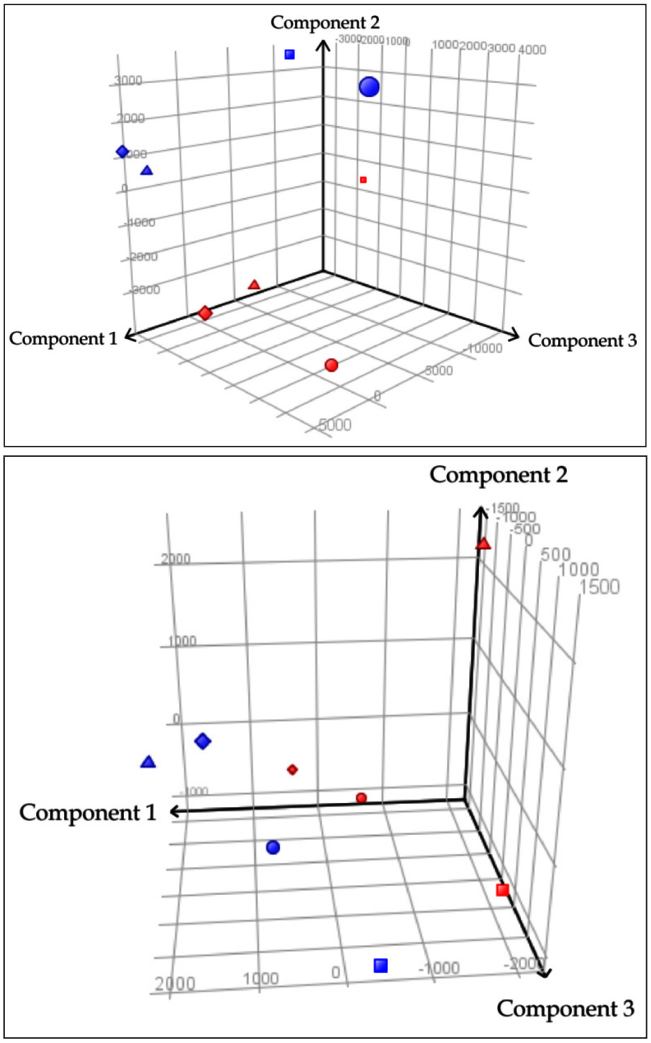


Figure 1. TLC plate with separated lipid standards (SM = Sphingomyelin, PS = Phosphatidylserine, PI = Phosphatidylinositol, PE = Phosphatidylethanolamine, PC = Phosphatidylcholine)

Results and Discussion

The analysis of the storage-induced changes of the RBC lipidome by means of HPLC/TOF-MS yielded highly significant differences between fresh blood and RBCs stored for 42 days ($p < 0.01$, t-test). In Figures 2 and 3, the PCA plots of untargeted results obtained by LC-TOF measurements in positive and negative mode are depicted and the samples of subjects 1-4 stored for 0 (red) and 42 days (blue) are clearly separated.



Figures 2 and 3. PCA plot of untargeted results obtained by LC-TOF measurements in positive mode. Subject 1: squares, subject 2: circles, subject 3: triangles, subject 4: diamonds. Red color represents fresh blood, blue color 42 days of storage.

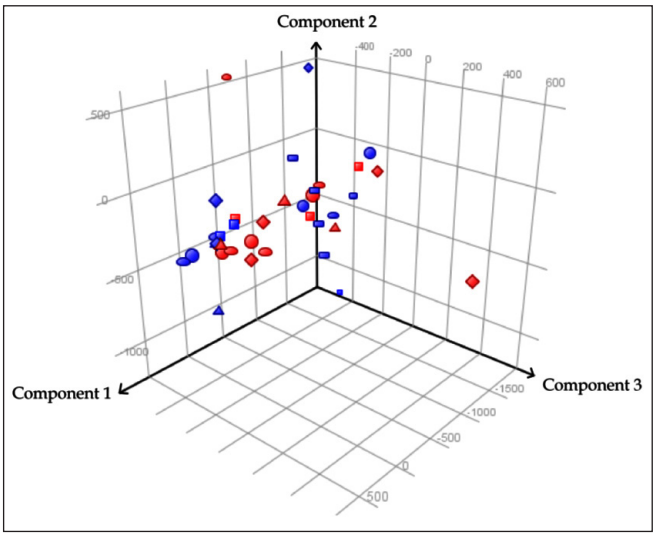


Figure 4. PCA plot of untargeted results obtained by GC-TOF measurements after TLC separation. Fraction of PC represented by squares, PE by triangles, PI by circles, PS by diamonds and SM by ovals. Red color represents fresh blood, blue color 42 days of storage.

However, for GC measurements no significant differences could be confirmed for any of the investigated lipid sub-classes (Figure 4).

In the last years, several studies focused on the characterization of RBC storage lesions and successfully described alterations of both membrane and cytosolic proteins [1, 3-7]. Thus, storage-induced changes of the RBC lipidome were to be expected. Both approaches presented in this study proved to be suitable for the analysis of the RBC lipidome and further investigations of the identified lipids will have to show if the observed alterations are sufficient for doping control purposes.

Conclusions

Within this study, two complementary approaches for the analysis of the RBC lipidome were presented and successfully used for the analysis of the RBC lipidome. By using HPLC/TOF-MS, highly significant differences between the lipidome of RBCs stored for 0 and 42 days were detected. Further evaluation of the data might result in the identification of lipid biomarkers for RBC storage lesions that could be used to provide direct evidence for the misuse of ABT following reinfusion in elite athletes.

References

[1] Walpurgis K, Kohler M, Thomas A, Wenzel F, Geyer H, Schänzer W, Thevis M (2012) Storage-induced changes of the cytosolic red blood cell proteome analyzed by two-dimensional difference gel electrophoresis (2D DIGE) and high resolution/high accuracy mass spectrometry. Proteomics 12(21), 3263-3272.

[3] Kriebardis AG, Antonelou MH, Stamoulis KE, Economou-Petersen E, Margaritis LH, Papassideri IS (2007) Progressive oxidation of cytoskeletal proteins and accumulation of denatured hemoglobin in stored red cells. J Cell Mol Med 11(1), 148-155.

[4] Wolfe LC, Byrne AM, Lux SE (1986) Molecular defect in the membrane skeleton of blood bank-stored red cells. Abnormal spectrin-protein 4.1-actin complex formation. J Clin Invest 78(6), 1681-1686.

[5] Wagner GM, Chiu DT, Qju JH, Heath RH, Lubin BH (1987) Spectrin oxidation correlates with membrane vesiculation in stored RBCs. Blood 69(6), 1777-1781.

[6] D'Amici GM, Rinalducci S, Zolla L (2007) Proteomic analysis of RBC membrane protein degradation during blood storage. J Proteome Res 6(8), 3242-3255.

[7] Walpurgis K, Kohler M, Thomas A, Wenzel F, Geyer H, Schänzer W, Thevis M (2013) Effects of gamma irradiation and 15 days of subsequent ex vivo storage on the cytosolic red blood cell proteome analyzed by 2D-DIGE and Orbitrap MS. Proteomics Clin Appl, 7(7-8), 561-570.

www.agilent.com

Published April 10, 2015
Publication number: 5991-5939EN

Agilent Technologies