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Whole blood glycerophospholipids in dried blood spots – a reliable marker for the fatty acid status

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ABSTRACT

Background: Whole blood total fatty acid analysis in capillary blood has recently been proposed for fatty acid status determination, but the accuracy of this method is affected by the fast turnover of triaclyglyceride (TAG) fatty acids, the individual hematocrit and postprandial state.
Method: An established method for the glycerophospholipid (GPL) analysis in plasma was adapted for the analysis of whole blood GPL and tested in a fat challenge test. Blood samples were collected from nine participants after receiving a standardised breakfast containing 42 g of sunflower oil. Whole blood GPL fatty acids were compared against whole blood total lipid, plasma TAG and phospholipid fatty acids.
Results: All fatty acid concentrations in dried blood samples showed a coefficient of variation <5.7%. The fat challenge test induced a significant increase of TAG fatty acid concentration (mean $\Delta = 42.3\% \pm 35.7$) and whole blood total fatty acid concentration (mean $\Delta = 5.2\% \pm 3.7$) whereas whole blood GPL fatty acids were hardly changed (mean $\Delta = 1.3\% \pm 1.6$).
Conclusion: Whole blood GPL fatty acids are a robust biological marker for the fatty acid status of fasted and non-fasted subjects. The influence of very recent dietary intake on whole blood GPL is smaller than on whole blood total lipids.

1. Introduction

Long-chain polyunsaturated fatty acids (LC-PUFA) such as arachidonic acid (AA), eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) have been associated with foetal and infantile development, cardiovascular function, and mental diseases (Fleith and Clandinin, 2005; Koletzko et al., 2008; Leaf, 2006).

The LC-PUFA status in humans is usually assessed by analysing the fatty acid pattern in plasma or red blood cell (RBC) phospholipids (PL) (Fekete et al., 2009). However, the collection of blood samples via venipuncture is not well accepted by many adults or infants and generally has to be performed under conditions of a clinical setting. In recent years, researchers developed methods which are less invasive but provide useful data on the fatty acid status (Armstrong et al., 2008; Bailey-Hall et al., 2008; Bicalho et al., 2008; Klingler and Koletzko, 2012; Marangoni et al., 2004; Min et al., 2011). Capillary blood collected by a finger prick has been established for the determination of whole blood fatty acids (Armstrong et al., 2008; Bailey-Hall et al., 2008; Galli et al., 2009; Marangoni et al., 2004; Metherel et al., 2009). Apart from being less invasive, this method is cost effective and does not demand the same effort to collect a blood specimen as by venipuncture. The lipids are extracted directly from dried blood spots on filter paper into solvents used for the analysis. However, due to the combined analysis of plasma and RBC lipids the significance of the results might be limited (Klingler and Koletzko, 2012). The specific fatty acid pattern of individual lipid fractions are different and their contribution to total analysed lipids varies with the individual hematocrit and postprandial state.

Another issue that has to be addressed is the storage of dried blood spots on filter paper over a long period. Studies have shown that despite using antioxidants such as butyl hydroxyl toluene (BHT) DHA contents in dried blood spots decline over time, presumably due to the large surface area of the filter paper used and hence a large oxygen exposure (Min et al., 2011). It should be underscored that opinions regarding the efficacy of BHT are varied (Ansar et al., 2013; Metherel et al., 2013a, 2013b; Quiroga et al.,

Abbreviations: DBS, dried blood spots.

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2015). Overall, the problem of DHA decay limits the use of dried blood spots for fatty acid analysis, if blood samples cannot be processed immediately.

In this study, a selective method for the analysis of glycerophospholipid fatty acids (GPL) was tested for analysis of dried whole blood samples. The method has been developed for the analysis of plasma GPL (Glaser et al., 2010) and was also successfully adapted to red blood cell and cheek cell GPL (Klem et al., 2012; Klingler et al., 2011). An advantage of the selective analysis of GPL might be that this lipid fraction is hardly affected by the postprandial state. To test this hypothesis, whole blood GPL fatty acids were analysed after a meal, which included 42 g linoleic acid rich sunflower oil. Studies have shown that this amount is sufficient to increase blood triacylglycerides (TAG) and PL significantly within a couple of hours after consumption (Dubois et al., 1998; Mekki et al., 2002).

The potential influence of different factors on storage and measurement of fatty acid concentrations in blood drops collected using filter paper was investigated previously (Armstrong et al., 2008; Gordon Bell et al., 2011; Metherel et al., 2013a, 2013b, 2012; Min et al., 2011). This focused on the impact of different drying conditions and storage over three months on degradation of AA and DHA in blood spots.

2. Materials and methods

2.1. Subjects

For method development, anonymised blood samples were used. The method was then applied in a fat challenge test performed in apparently healthy adult volunteers (three males and six females). Characteristics of the nine participants are shown in Table 1. The Ethical committee of the University of Munich Medical Centre approved the study (251-10). Written informed consent was obtained from all participants prior to trial commencement.

2.2. Fat challenge test

Participants received a standardised breakfast after overnight fasting. The meal contained a muffin, a chicken sandwich with mayonnaise and a glass of orange juice. In total, the meal contained 3784 kJ, 25 g of protein, 97 g of carbohydrates and 45 g of fat (42 g fat derived as triglyceride from sunflower oil). The content of various fatty acids in the meal was as follows: C16:0–3.4 g, C18:0–1.3 g, C18:1n-9–11.1 g and C18:2n-6–26.4 g). Blood samples were collected before the test meal intake and then hourly over seven hours. The participants were not allowed to eat or drink within this time, except for water.

2.3. Blood sampling

Blood samples used for method development were collected after an overnight fasting period from the antecubital vein in 7.5 ml EDTA monovettes (Sarstedt, Nümbrecht, Germany). Blood samples were kept on ice until centrifugation (1000 × *g*, 10 min, 4 °C), which was done within 2 h after sampling. All samples were prepared immediately for GPL fatty acid analysis unless otherwise stated. Capillary blood was obtained by finger prick using a sterile Terumo FineTouch® lancet (Terumo Corporation, Tokyo, Japan). The first blood drop was wiped off with sterile gauze, and blood was dropped on the pre-labelled areas of Protein Saver 903 Cards® (Whatman, Maidstone, UK). The filter paper was dried for 3 h under vacuum prior to fatty acid analysis.

Table 1
Characteristics of subjects participating in the fat challenge test (n = 9).

	mean	SD
Age [years]	33.4	11.0
BMI [kg/m^2]	22.4	2.6
Cholesterol [mg/dl]	197	25
Triglycerides [mg/dl]	90	48
LDL [mg/dl]	103	40
HDL [mg/dl]	79	29
Ratio LDL/HDL	1.7	1.2

2.4. Drying procedures for blood spots on filter paper

Different drying procedures were tested to determine the stability of LC-PUFA on filter paper: drying at air, drying with compressed air or nitrogen, and drying in vacuum. For each procedure, filter paper spots with 60 μl of venous whole blood were used. The circles with non-dried blood were cut with scissors into four small pieces. Samples, which were exposed to air were kept on small plastic trays. Samples, dried with compressed air or nitrogen were kept in open 4 ml brown glass bottles under a continuous gas flow. Samples dried in vacuum were placed in an evacuated exsiccator. The samples were exposed for 24 h to the respective treatment and sampling was done hourly up to 6 h and once again after 24 h.

2.5. Long-term storage of dried blood spots

Venous whole blood samples of subjects participating in the fat challenge test (n = 9) were collected on Protein Saver 903 Cards®. The initial sample was analysed immediately before drying. The samples for storage were dried under vacuum for 3 h, put in plastic bags, closed and frozen at −80 °C. Contents (mol%) of AA and DHA of the blood spots were measured monthly over three months and compared against baseline and each other.

2.6. Analysis of whole blood total fatty acids

Venous whole blood (100 μl) was haemolysed by the addition of 100 μl distilled water. A mixture of 1100 μl isopropanol (+50 mg/l BHT) and 700 μl chloroform containing 50 μl internal standard A (25 μg each of pentadecanoic acid, tripentadecanoin, phosphatidylcholine (PC) dipentadecanoyl, and cholesteryl pentadecanoate, dissolved in 100 μl methanol/chloroform, Sigma Aldrich, Taufkirchen, Germany) was added under continuous shaking on a Vibrax shaker (IKA, Stauffen, Germany). The suspension was treated in an ultrasound water bath (120 W, 35 kHz) at room temperature for 5 min followed by centrifugation (3030 × *g*, 10 min, 4 °C). Subsequently, the supernatant was transferred into a 4 ml brown glass vial and solvents were evaporated under a continuous nitrogen flow. The lipid extract was re-dissolved in 1.5 ml 3 M methanolic HCl (Supelco, Bellefonte, USA) and fatty acid methyl ester (FAME) synthesis took place in a sealed vial at 85 °C for 45 min. After cooling, the solution was neutralised with a tip of a spatula of a dry mixture of sodium carbonate, sodium hydrogen carbonate and sodium sulphate (1:2:2 by weight). FAME were extracted twice into 1 ml hexane, the extracts were combined and hexane was evaporated under nitrogen flow. Subsequently, FAME were re-dissolved in 50 μl hexane containing BHT (2 g/l) and stored at −20 °C until gas chromatographic (GC) analysis.

2.7. GPL fatty acid analysis of venous whole blood

A volume of 60 μl whole blood was haemolysed with 140 μl distilled water. Methanol (1350 μl) and 50 μl of internal standard B