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Influence of collection tubes during quantitative targeted metabolomics studies in human blood samples

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ABSTRACT

Background: Plasma and serum are the most widely used matrices in clinical studies. However, some variability in absolute concentrations of metabolites are likely to be observed in these collection tubes matrices.
Methods: We analyzed 189 metabolites using the same protocol for quantitative targeted metabolomics (LC-MS/MS AbsoluteIDQ p180 Kit Biocrates) in three types of samples, serum, plasma EDTA and citrate, of 80 subjects from the Cooperative Health Research In South Tyrol cohort (40 healthy elderly and 40 healthy young).
Results: The concentration levels were higher in serum than citrate and EDTA, in particular for amino acids and biogenic amines. The average Pearson's correlation coefficients were however always higher than 0.7 for these two classes of metabolites. We could also demonstrate that blank EDTA vacutainer tubes contain a significant amount of sarcosine. Finally, we compared the metabolome of young people against elderly subjects and found that the highest number of metabolites significantly changing with age was detected in serum.
Conclusion: Serum samples provide higher sensitivity for biomarker discovery studies. Due to the presence of spurious amount of sarcosine in vacutainer EDTA tubes, plasma EDTA is not suitable for studies requiring accurate quantification of sarcosine.

1. Introduction

Metabolomics is the system level analysis of metabolism driven by the comprehensive identification and quantification of small-molecules in biological systems.

Metabolomics closely reflects the phenotype, since it integrates individual's genetic background, ageing and lifestyle. Metabolomics analysis can be performed in different sample types, such as tissues [1], cells [2–5] and biofluids [6]. In recent years the simultaneous development of biobanking and high-throughput technologies has enabled a new generation of successful large-scale cohort studies where metabolomics can been applied [7–13].

Biobanks can store and distribute to researchers a large amount of blood products. The blood components, plasma and serum, are the most widely used matrices in clinical studies, although there are alternative options of blood collection such as dried blood spots (DBS) [14] and volumetric absorptive microsampling (VAMS) [6] that offer the opportunity of remote collection, without the presence of trained staff. Both plasma and serum are separated from whole blood via centrifugation. Serum collection tubes contain a separation gel and the blood is separated after coagulation has occurred; on the other hand, plasma collection tubes contain anticoagulants, such as EDTA, heparin or citrate, which prevent blood clotting.

Selecting the correct matrix for biochemical analysis is of paramount importance in order to avoid improper diagnosis [15]. Blood collection tubes are often an under-recognized variable in the pre-analytical phase of clinical studies, and it is thus important to evaluate the influences of these matrices during metabolomics analysis [16–18].

Abbreviations: LC-MS, liquid chromatography mass spectrometry; CHRIS, Cooperative Health Research In South Tyrol; FIA, flow-injection analysis; SOPs, standard operating procedures; UHPLC, ultra-high performance liquid chromatography; HILIC, hydrophilic interaction liquid chromatography; PCA, principal component analysis; PC 1, first principal component; PC 2, second principal component; PC 4, fourth principal component; QC, quality control; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; C18:1, octadecenoylcarnitine; PC aa C40:3, 1,2-diacyl-*sn*-glycero-3-phosphocholine 40:3

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Liquid chromatography mass spectrometry (LC-MS) is one of the most popular platform used for metabolomics studies. In literature several reports have explored the influence of collection tubes in metabolic profiling studies, but almost all of them compared untargeted data and not quantitative data [19–21].

The use of commercially available kits for quantitative targeted metabolomics has become popular since it simplifies inter-laboratory comparison and the exchange of data coming from different studies [22]. Among these kits, the Biocrates AbsoluteIDQ® p180 kit, which can be used on a variety of LC-MS/MS instruments, has already been applied to many studies of human serum and plasma, including several large-scale prospective cohort studies [7–9, 11–13, 23]. A recent study compared the influence of plasma EDTA and serum in targeted metabolomics using a similar commercially available kit (Biocrates AbsoluteIDQ® p150) [24].

The aim of this study was to investigate the influence of collection tubes for quantitative targeted metabolomics studies in samples collected during the Cooperative Health Research In South Tyrol (CHRIS) study in order to determine which of these is more suitable for large scale metabolomics studies of the cohort. CHRIS is a population-based study that addresses genetic and molecular basis of common chronic conditions and their interaction with life style and environment in the general population. Several biospecimens, including serum, plasma EDTA and plasma citrate, were collected from participants and stored at the CHRIS biobank [25].

For that, we selected 80 apparently healthy subjects from the CHRIS cohort. These 80 individuals were not known to suffer from any physical or mental disease and were analyzed using the Absolute IDQTM p180 Kit resulting in the measurement of 189 metabolites in each of three investigated matrices.

2. Materials and methods

2.1. Study design and sample collection

The CHRIS study has been described in detail previously [25]. Briefly, the study was carried on in Venosta valley, South Tyrol, Italy. Recruitment was gradual and all 28,000 adults resident in the selected study area were invited to participate, in groups of up to 10 participants per working day.

We included in this study 80 participants enrolled from the first 4979 participants who joined the study between August 24th, 2011 and July 15th, 2014.

A total of 40 independent individuals were selected with age lower than 35 years and assigned to the “young” group. The other 40 independent subjects were selected with age higher than 60 years and assigned to the “elderly” group. All selected individuals were unrelated and free from any diagnosed cardiovascular, metabolic or other chronic disease and did not take any medication on a regular basis. Both groups were sex-matched. In addition, to exclude any potential influence of seasonality, the selection of individuals required participation dates from the same season [26].

Blood was collected at the recruitment center after overnight fasting, and taken in the early morning within a short time interval. We followed WHO guidelines for use of anticoagulants in diagnostic laboratory investigations and stability of blood, plasma, and serum samples (WHO/DIL/LAB/99.1 Rev.2 on http://apps.who.int/iris/handle/10665/65957). To ensure measurements' reliability and accuracy, trained nurses performed pre-analytical processing immediately after the drawing. Serum, EDTA and Citrate Vacuette® vacutainer collection tubes were provided by Greiner Bio-One (Kremsmünster, Austria). All vacutainers were inverted five times immediately after blood drawing to mix the anti coagulant or the clot activator with the whole blood.

Serum tubes were allowed to clot for 30 min at room temperature and centrifuged for 15 min at 1500 ×g at RT. Blood tubes with EDTA and citrate were further mixed after blood drawing on a sample rotator for 10 min at room temperature. EDTA tubes had no further treatment, whereas citrate vacutainers were centrifuged for 15 min at 1500 ×g at RT.

EDTA, citrate, and serum tubes from the same day were stored altogether in a transportation bag at room temperature and shipped to the laboratory/biobank. The temperature range during transportation was fixed and monitored through the entire recruitment period. Aliquots of 120 µL were then obtained for serum, plasma EDTA and plasma citrate samples and stored at −80 °C at the Biobank of Bozen/Bolzano until the analysis.

All participants gave written informed consent in accordance with the Declaration of Helsinki. The study received ethical approval by the Ethical Committee of the Healthcare System of the Autonomous Province of Bolzano.

2.2. Chemicals

Acetonitrile was purchased from VWR International (Radnor, PE, USA). Methanol was obtained from Sigma–Aldrich (Seelze, Germany). Water was obtained from a Milli-Q water purification system equipped with LC-pak® polisher (Millipore, USA). All solvents were of LCMS analytical grade or higher purity. NIST plasma EDTA QC sample, Standard Reference Material® 1950 was purchased from NIST (Gaithersburg, MD, USA).

2.3. Liquid chromatography mass spectrometry

Targeted analysis was performed using the Biocrates AbsoluteIDQ® p180 kit with an ultra-high-performance liquid chromatography (UHPLC) (Agilent 1290, Agilent Technologies, Santa Clara, CA, USA) coupled to a Q-Trap mass spectrometer (MS) (QTRAP 6500, Sciex, Foster City, CA, USA).

Sample preparation and analysis were performed according to the manufacturer's protocol. In brief, the sample processing procedure was performed on a single sample and utilized a 96-well plate design where both sample derivatization and analyte extraction were performed. The kit requires 10 µL of sample and provides human plasma based quality controls in 3 concentration levels (low, medium, high) which can be used for quality control purposes and batch normalization. Once processed, each sample undergoes to two separate MS-based analytical runs. A total of 189 metabolites are included in the kit, 43 metabolites are measured by UHPLC-MS/MS and 146 metabolites by flow-injection analysis (FIA)-MS/MS. Isotope-labelled and chemically homologous internal standards are used for quantification providing a total of 56 analytes fully validated as absolutely quantitative. The UHPLC-MS/MS run provide fully validated absolute quantification of 43 metabolites, including 21 amino acids and 22 biogenic amines, with the use of external calibration standards in seven different concentrations and isotope labelled internal standards for most analytes. The remaining 146 metabolites, including 40 acylcarnitines, 90 glycerophospholipids, 15 sphingolipids and 1 sum of hexoses, were analyzed by FIA-MS/MS, using a one point internal standard calibration with representative internal standards (9 isotope-labelled acylcarnitines, 1 isotope-labelled hexose, 1 non-labelled lyso-PC, 2 non-labelled PCs, 1 non-labelled SM, a total of 14 internal standards). 12 acylcarnitines and the sum of hexoses are fully validated as quantitative while for the remaining metabolites analyzed by FIA-MS/MS the kit provides a “semi-quantitative” measurement due to the lack of commercially available specific internal standards. Several lipids analyzed in the present kit represent the total concentrations of possible isobars and structural isomers.”

Sarcosine confirmation was achieved by running the content of blank serum, plasma citrate and plasma EDTA vacutainer tubes with our untargeted MS-based workflows [6]. In brief, 500 µL of water:-methanol (1.1) were added in each vacutainer tube, vortexed 4000 RPM for 5 min, centrifuged at 4300 RPM for 10 Minutes at 20 °C and