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Specimen Collection and Handling

Standardization of Blood Sample Collection

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Summary

Preanalytical variables can alter the analysis of blood-derived samples. Prior to the analysis of a blood sample, multiple steps are necessary to generate the desired specimen. The choice of blood specimens, its collection, handling, processing, and storage are important aspects since these characteristics can have a tremendous impact on the results of the analysis.

The awareness of clinical practices in medical laboratories and the current knowledge allow for identification of specific variables that affect the results of a proteomic study. The knowledge of preanalytical variables is a prerequisite to understand and control their impact.

Key Words: blood; plasma; serum; proteomics; specimen; preanalytical variables.

1. Introduction

Proteomic analysis of blood specimens by semi-quantitative multiplex techniques offers a valuable approach for discovery of disease or therapy-related biomarkers ***(1,2)***. Based on reproducible separation of proteins by their physical–chemical properties in combination with semi-quantitative detection methods and bioinformatic data analysis, proteomics allows for sensitive measurement of proteins in blood specimens ***(3)***. Blood can be regarded as a complex liquid tissue that comprises cells and extracellular fluid ***(4)***. The choice of a suitable specimen-collection protocol is crucial to minimize artificial processes (e.g., cell lysis, proteolysis) occurring during specimen collection and preparation ***(5)***. Preanalytic procedures can alter the analysis of blood-derived

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samples. These procedures comprise the processes prior to actual analysis of the sample and include steps needed to obtain the primary sample (e.g., blood) and the analytical specimen (e.g., plasma, serum, cells). Legal or ethical issues (e.g., importance of informed consents) or potential risks of phlebotomy (e.g., bleeding) are not covered in this article.

1.1. Collection of Blood Samples

It has been reported that the most frequent faults in the preanalytical phase are the result of erroneous procedures of sample collection (e.g., drawing blood from an infusive line resulting in sample dilution) ***(6)***. The design of blood collection devices may aid in correct sampling: evacuated containers sustain the draw of accurate quantity of blood to ensure the correct concentration of additives or the correct dilution of the blood, such as in the case of citrated plasma. The speed of blood draw is also controlled and restricts the mechanical stress. The favored site of collection is the median cubital vein, which is generally easily found and accessed. As such, it will be most comfortable to the patient, and should not evoke additional stress. Preparation of the collection site includes proper cleaning of the skin with alcohol (2-propanol). The alcohol must be allowed to evaporate, since commingling of the remaining alcohol with blood sample may result in hemolysis, raise the levels of distinct analytes, and cause interferences. The position of the patient (standing, lying, sitting) can affect the hematocrit ***(7)***, and hence may change the concentration of the analytes. Tourniquet should be applied 3–4 inches above the site of venipuncture and should be released as soon as blood begins flowing into the collection device. The duration of venous occlusion (>1 min) can affect the sample composition. Prolonged occlusion may result in hemoconcentration and subsequently increase the miscellaneous analytes, e.g., total protein levels. Blood should be collected from fasting patients in the morning between 7 and 9 a.m., because ingestion or circadian rhythms can alter the concentration of analytes considerably (e.g., total protein, hemoglobin, myoglobin).

1.2. Characteristics of Serum and Plasma Specimens

Serum is one of the most frequently analyzed blood specimens. The generation of serum is time consuming and associated with the activation of coagulation cascade and complement system. These processes influence the composition of the samples, because they result in cell lysis (e.g., thrombocytes, erythrocytes). As a consequence, the concentration of components in the extracellular fluid, such as aspartate-aminotransferase, serotonin, neuron-specific enolase, and lactate-dehydrogenase, are increased ***(8)***. On the other hand, degradation of the analytes (e.g., hormones) may occur faster ***(9)***. On the

proteomic level, more peptides and less proteins are observed in serum when compared to plasma ***(10,11)***.

Consequently, the activation of clotting cascades necessary to generate serum can lead to artefacts. A reason to use serum as a specimen is based on the notion that the proteome or peptidome of serum may reflect biological events ***(12)***. Post-sampling proteolytic cleavage products have been proposed as biomarkers, and it has been further suggested that serum peptidome is of particular diagnostic value for the detection of cancer ***(13)***. However, it has been reported that more protein changes occur in serum than in plasma ***(14)***. Thus, it can be expected that the reproducibility of such *ex vivo* proteolytic events is comparatively low.

In contrast to serum, citrate and EDTA inhibit coagulation and other enzymatic processes by chelate formation with ions, thereby inhibiting ion-dependent enzymes. This is in contrast to heparin, which acts through the activation of antithrombin III. The main concern associated with heparinized plasma for proteomic studies is that it is a poly-disperse charged molecule that binds many proteins non-specifically ***(15,16)***, and may also influence separation procedures and mass spectrometric detection of peptides and small proteins due to its similar molecular weight ***(17)***.

The sampling of plasma is less time consuming than the acquisition of serum. Separation of the cells and the liquid phase can be performed subsequently to sample collection since no clotting time is required (30–60 min). In comparison to serum, the amount of plasma generated from blood is approximately 10 to 20% higher. Additionally, the protein content of plasma is also higher than in serum, because of the presence of clotting factors and associated components. Furthermore, proteins may be bound to the clot, resulting in a decrease of protein concentration.

1.3. Processing of Blood Samples

A quick separation of cells from the plasma is favorable, since cellular constituents may liberate substances that alter the composition of the sample. Generally, it is recommended that plasma and serum be centrifuged with 1300–2000×*g* for 10 min within 30 min from the collection of the sample. The temperature should generally be 15–24°C ***(18)***, unless recommended differently for distinct analytes like gastrin or A-type natriuretic peptide. Processing at 4°C appears to be attractive, because enzymatic degradation processes are reduced at low temperatures. However, platelets become activated at low temperatures ***(19)*** and release intracellular proteins and enzymes, which affect the sample composition. Thus, processing at low temperatures is safe only after thrombocytes have been removed. Since one centrifugation step may be insufficient for

depletion of platelets below 10 cells/nL, a second centrifugation step (2500×g for 15 min at room temperature) or filtration step may be required to obtain platelet-poor plasma. This procedure is applicable only to plasma since the platelets in serum are already activated.

1.4. Protease Inhibitors

Protease inhibitors would be attractive, but commonly used protease cocktails may introduce difficulties due to interference with mass spectrometry and formation of covalent bonds with proteins, which would result in shifting the isoform pattern ***(20)***. Protease inhibitors have been considered and investigated as additives in proteome research to prevent or slow down proteolytic processes and thereby provide a means of more sensitive detection of markers in blood ***(21)***.

Even though protein integrity has been shown to be maintained by the addition of 15 commercially available protease inhibitors, the usefulness of protease inhibitors in overall protein stabilization of blood samples remains to be investigated in more detail ***(22)***. The presence of certain protease inhibitors in whole blood is toxic to live cells. Stressed, apoptotic, or necrotic cells release substances, and it may be argued that this affects the composition of serum or plasma until the cellular and soluble factions of blood are separated. However, careful selection of an appropriate protease inhibitor may solve this problem.

2. Materials

1. Twenty gauge needles and an appropriate adapter (e.g., Sarstedt, Nümbrecht, Germany) or a Vacutainer system (BD Bioscience, Franklin Lakes, USA).
2. Alcohol (2-propanol) in spray flask.
3. Swabs.
4. Examination gloves.
5. Tourniquet or sphygmomanometer.
6. Blood collection tubes (e.g., Sarstedt).
7. Centrifuge with a swinging bucket rotor (e.g., Sigma 4K15, Sigma Laborzentrifugen, Osterode, Harz).
8. A 10-mL syringe equipped with a cellulose acetate filter unit with 0.2 μm pore size and 5 cm^2 filtration area (e.g., Sartorius Minisart, Sarstedt).
9. 2 mL cryo-vials.
10. Pipette and tips.

3. Methods

1. Venipuncture of a cubital vein is performed using a 20-gauge needle (diameter: 0.9 mm, e.g., butterfly system max. tubing length: 6 cm). If tourniquet is applied, it should not remain in place for longer than 1 min (risk of falsifying results due to

hemoconcentration). As soon as the blood flows into the container, the tourniquet has to be released at least partially. If more time is required, the tourniquet has to be released so that circulation resumes and normal skin color returns to extremity.

- Prior to blood collection for proteomic analysis, blood is aspirated into the first container (e.g., 2.7 mL S-Monovette, Sarstedt, Nümbrecht, Germany). This is done to flush the surface and remove initial traces of contact-induced coagulation. This sample is not useful for analysis.
- Afterward, blood is drawn into a standard EDTA or citrate-containing syringe (e.g. 9 mL EDTA-Monovette, Sarstedt, Nümbrecht, Germany). Depending on ease of blood flow, several samples can be collected. Free flow with mild aspiration should be assured to avoid haemolysis.

2. After venipuncture, plasma is obtained by centrifugation for 10 min at $2000\times g$ at room temperature. Centrifugation should start within 30 min after blood collection. The resulting plasma sample may now be separated from red and white blood cells in an efficient and gentle way. Nevertheless, a significant number of platelets (~25%) are still present in the sample. This requires an additional preparation step.
3. For platelet depletion, one of the following procedures has to be undertaken directly after **step 2**:

- Platelet removal by centrifugation: The plasma sample is transferred into a second vial for another centrifugation for 15 min at $2500\times g$ at room temperature. After centrifugation, the supernatant is transferred in aliquots of 1.5 mL into cryo vials.
- Platelet removal by filtration: Plasma aliquots of 1.5 mL resulting from **step 2** are transferred into 2-mL cryo vials using a 10-mL syringe equipped with a cellulose acetate filter unit with 0.2 μm pore size and 5 cm^2 filtration area (e.g., Sartorius Minisart®, Sartorius, Göttingen, Germany). Filtration requires only gentle pressure.

4. Samples are transferred to an –80°C freezer within 30 min. Storage is at –80°C. Transport of samples is done on dry ice.

4. Notes

4.1. Frequently Made Mistakes

4.1.1. Blood Withdrawal

- The patient was not fasting (i.e., had taken food prior to sampling).
- The blood was drawn from an infusive line.
- The blood was drawn in a wrong position (e.g., supine, upright).
- The consumables used were different than those recommended.

- The expiry date of consumables was already reached.
- The tubes were not properly filled.
- The tubes were agitated vigorously (instead of gentle shaking to dissolve the anticoagulant).
- The blood sample tubes were not consistently kept at room temperature.
- The sample tubes were put on ice or in a refrigerator.

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4.1.2. Lab Handling

- Centrifugation was delayed more than 30 min after blood withdrawal.
- A cooling centrifuge was adjusted below room temperature.
- The centrifugation speed was wrong (e.g., rounds per minute were set instead of g-force).
- The centrifugation time was wrong.
- The removal of blood plasma by pipetting was done without proper caution. Consequently, the buffy coat or the red blood cells were churned up.
- The second centrifugation of recovered plasma samples was delayed after first centrifugation.

4.1.3. Storage of Samples

- The storage of samples was delayed.
- The storage temperatures were above –80°C.
- The labeling of sample containers was unreadable or confusable.
- The attachment of labels to the sample containers was not proper during storage or handling resulted in loss of labels.

4.1.4. General Recommendations

- A proper first centrifugation should produce a visible white blood cell layer (buffy coat) between red blood cells and plasma. If not, centrifugation speed or time may be wrong.
- One should discard plasma that is icteric or exhibits signs of haemolysis. One should check with an expert if this was due to that particular disease.

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