Considerations for the Use of Blood Plasma and Serum for Proteomic Analysis

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Citation

Abstract

Blood and serum are frequently used as samples for proteomic analysis. A consideration of the literature suggests that the differences between serum and plasma as samples are not appreciated by many investigators. There also appears to be a lack of understanding of the issues critical for the processing of plasma and serum samples for analysis. The major difference between plasma and serum is the removal of fibrinogen and associated proteins such as high-molecular weight von Willebrand factor and the variable addition of cellular secretion products as a result of the coagulation process. Factors influencing plasma quality include anticoagulant selection, processing, and storage. Factors influencing serum quality include collection container and clot retraction time. Various approaches for the removal of high abundance proteins such as albumin and immunoglobulin G will be discussed. While it is clear that the removal of these high abundance proteins provides for the analysis of otherwise obscured low-abundance proteins, studies have not been performed to document that some low-abundance proteins are also not removed by these techniques Furthermore, most studies do not have enough "normal" subjects to provide a statistically sound control value. Plasma and serum can be valuable samples for proteomic analysis but the preparation of the samples must be suitable for regulatory validation. Recommendations are presented for consideration.

INTRODUCTION

Blood is an extremely popular source for biological samples for proteomic analysis leading to the identification of biomarkers. Blood samples are reasonably easy to obtain, the samples are technically and psychologically easy to process (for example, feces, while easy to obtain, generally is considered difficult to process because of cultural reasons), and samples are mostly considered homogeneous when compared to saliva or urine both of which are somewhat compositionally dependent on fluid flow rates. There is also a large amount of data on "normal" composition since blood is used so frequently for diagnostic purposes [1, 2, 3]. Palkuti [2] presents a good summary of the establishment of normal. A normal range (reference interval) must be established for each analyte (biomarker); this is value expected for 95% of the population. Thirty individuals are required for the establishment of a normal value. While there are some proteomic studies [4] which clearly meet this criteria, most studies do not and the argument is presented that such information is not necessary [5]. It is noted that there is one major study on the human serum proteome where the sample was obtained from a single individual [6]; I can't imagine a study being performed on a single normal subject with

another technology which would be seriously considered. While it might be difficult, but not impossible, to secure sufficient tissue samples for the definition of normal, it is much easier with blood samples.

The above pretty much takes care of the "good news" about the use of blood as a source for the identification of biomarkers. One of the most critical issues in proteomic analysis is sample preparation [7, 8]. The initial blood sample is removed from the circulatory system via venipuncture [9, 10]. If the sample is withdrawn in the presence of an anticoagulant and centrifuged to remove cellular elements, a plasma sample is obtained; in the absence of anticoagulant, the blood clots and can be centrifuged immediately to remove the fibrin clot and cellular elements or allowed to stand for period of time and clot retraction occurs [11, 12]. As noted below, it possible to withdraw blood through a resin-containing device which deplete the blood of calcium precluding coagulation [13]. The addition of corn trypsin inhibitor to the blood as withdrawn [14, 15] greatly slows the process of blood coagulation would permit facile removal of the cellular elements and transfer, for example, into urea-detergent prior to two-dimensional gel electrophoresis. It is also likely that the presence of the common protease

inhibitor cocktails would also sufficiently inhibit coagulation to permit the processing of plasma samples. The most common anticoagulants are EDTA (ethylenediamine tetraacetic acid) or sodium citrate and heparin; EDTA and citrate function as anticoagulants by chelating calcium ions while heparin "activates" antithrombin. While comparative studies in proteomics are not available, EDTA should be considered preferable to citrate as an anticoagulant [9].

SERUM IS, OF COURSE, NOT PLASMA

This fact seems to be ignored by a large number of investigators who consider serum and plasma to be identical. Serum is qualitatively different from plasma in that the bulk of the fibrinogen has been removed by conversion into a fibrin clot together with the platelets which have either been physically bound in the fibrin matrix or activated to form aggregates or both. Varying amounts of other proteins are removed into the fibrin clot either by specific or non-specific interactions. There are a number other changes [16] including the formation of protease-serpin complex and protein fragments such as D-dimer and prothrombin fragment 1. Contrary to some statements in the literature [17], many of the coagulation factors such as factor IX, factor X, and factor XI are retained in serum. As an example, consider factor VII/VIIa which was originally described as serum prothrombin conversion accelerator. Some the coagulation factors such as factor IXa are present in the precursor form (factor IX), the activated enzyme (factor IXa), and as a complex between the activated enzyme and antithrombin III. The protein concentration of serum is less than that of plasma [18, 19]. Lum and Gambino [18] reported an average value of 7.45 gm/dL for heparinized plasma versus an average value of 7.21 gm/dL for serum. The authors suggest that the difference is largely due to the removal of fibrinogen. These investigators did observe an increase in albumin in serum. A similar difference in mean protein concentration between serum (7.29 gm/dL) and plasma (7.58 gm/dL) was observed by Ladenson and coworkers [19]. These investigators also reported a higher concentration for albumin in serum.

In the process of whole blood coagulation, the cellular elements (erythrocytes, leukocytes, platelets) can secrete components. In particular, platelets contribute a variety of components to blood serum [20,21,22]. Vascular endothelial growth factor (VEGF) is an excellent example [23, 24]. In one study [23], normal individuals had a serum concentration of 250 pg/mL with a plasma concentration of 30 pg/mL; breast cancer patients with thrombocytosis had a median VEGF

concentration of 833 pg/mL compared to 249 pg/mL in other patients. Both studies suggest that platelets can contribute to VEGF levels in both plasma and serum but more markedly so in serum. It is suggested that the immediate separation of plasma or serum from the cellular elements provide optimal analyte stability [25]. Other investigators have observed an increased in potassium in serum [26, 27]. The time of contact of serum with the cellular elements will variably influence composition but sufficient time must be allowed to permit completion of fibrin formation. One aspect that is frequently ignored is that the quality of the in vitro blood clotting process in the nature of the container; there is a marked difference in serum obtained from clotting in plastic versus clotting in glass [28].

An alternative for sample preparation which, to the best of my knowledge, has not been explored for proteomic research is the defibrination of plasma with Bothrops jararraca venom (reptilase)[29, 30, 31]. Defibrinated plasma is not equivalent to serum. There are multiple anticoagulants available for the collection of blood plasma including citrate and heparin; the choice of anticoagulant and temperature can influence storage stability of the analyte [31, 33, 34, 35, 36, 37]. The difference between plastic and glass for the collection of serum is briefly discussed above. The material nature of the storage containers can also influence stability [38]. My personal experience suggests that the time between venipuncture and freezing, process/storage containers, centrifugation speed, and the temperature of storage are the most critical variables for plasma. Critical process variables for serum are process/storage containers, time of clot retraction/removal of the fibrin clot with associated platelets and other cellular elements, centrifugation speed, and temperature of storage.

The take-home message is that there are many factors other than the underlying biology which can influence a blood sample. It is difficult, if not impossible to eliminate these factors; the best that one can do is to very carefully document the conditions of blood processing. It is strongly recommended that a Standard Operating Procedure (SOP) be established for the process of obtaining a blood sample. It is only by doing this that one is able to assure reproducibility of samples and to allow some rationale comparison of data from various laboratories. Until that is accomplished, data analysis such as that recently presented by Anderson and others [17, 39] is interesting but of questionable value; while there may well be greater than a million discrete protein species in a plasma or serum sample, the significance of

such compositional heterogeneity is not clear. In particular, there is concern regarding the role of processing in the production of such heterogeneity. Likewise, the suggestion by Henschen-Edman [40] that there are a million discrete forms of fibrinogen may well be accurate but again the significance of such heterogeneity remains to be demonstrated. On the other hand, there is clear evidence that there are changes in fibrinogen during acute coronary syndrome as assessed by proteomic analysis [41] and there are significant changes in fibrinogen in human plasma after methylene blue photo-treatment [42] again as assessed by proteomic analysis. Finally, the longer the $t_{1/2}$ of a plasmas protein, the greater the chance of in vivo modification including oxidation and glycation which would introduce heterogeneity.

The next problem with the use of blood as a sample for proteomic analysis is the dynamic qualitative and quantitative range of proteins. While the dynamic range of protein concentration in plasma or serum cannot be precisely stated, it is clear that it is at least 10^6 and likely larger [6]. Care does need to be taken in this consideration as there are (1) intrinsic plasma proteins ranging from albumin at the high end (mg/mL) to blood coagulation factors such as factor VIII at the low end (μg/mL) giving a dynamic range of at least 10^4, (2) proteins and peptides such as insulin which are being transported by the circulatory system, and (3) proteins/peptides such as glutamic/oxaloacetic transaminase (SGOT)(liver disease) and troponin (myocardial infarction) which are released by tissues/cells as part of the pathological response. There are also substantial size heterogeneity in plasma with smaller proteins such as the vitamin K-dependent proteins which have molecular weights in 50 Kda range, fibrinogen which is approximately 340 Kda to von Willebrand Factor in the 10-20 million dalton range [43]. Considering this heterogeneity, prefractionation is essential for the proteomic analysis of blood. At the onset of the following discussion, it is recommended that free boundary electrophoresis [44, 45, 46, 47, 48, 49] be used for plasma/serum prefractionation. The bulk of the plasma proteins are comprised of several proteins and their isoforms, albumin, immunoglobulins, alpha-1-antitrypsin, fibrinogen, and haptoglobin. The presence of large amounts of these proteins creates technical difficulties for identifying minor components. One of these proteins, fibrinogen, is absent from serum creating an advantage as compared to plasma; other proteins are also missing in serum. Most efforts have been directed at methods for the removal of albumin and immunoglobulin as they are present in the highest concentrations (Table 1) [43].

Figure 1

Table 1: Some Characteristics of Major Human Plasma Proteins

Protein	Concentration (mg/mL)	M.W. (kDa)
Albumin	50	67
Immunoglobulin G	15	150
Fibrinogen	3	340
Haptoglobin	2.0	100
α₁-Antitrypsin	2.7	54
Immunoglobulin A	2.6	160
α₂-Macroglobulin	2.7	800
Blood Coagulation Factor VII	0.0005	50

These approaches use affinity matriceswhich remove a good portion but not all of the proteins; more problematical is that these columns also remove other components of plasma/serum by "non-specific" binding. Since most studies don't have a specific target protein, it is not possible to know whether a biomarker of interest is lost on the removal of albumin or immunoglobulin. It is less likely that components would be lost during the free boundary electrophoresis prefractionation step. The use of isoelectric membranes in a technique referred to as isoelectric trapping [50] has potential [51, 52] for the fractionation of plasma or serum. This should allow the preparation of relatively clean samples for subsequent analysis with the caveat that there might be protein solubility problems at the isoelectric point.

Affinity binding to Cibacron blue dyes [53, 54, 55] has been widely used for the depletion of albumin in plasma/serum samples prior to proteomic analysis [7, 56, 57, 58, 59, 60, 61]. Cibacron blue has also been used for the removal of albumin from cerebrospinal fluid [58, 60]. The binding of albumin to cibacron blue dyes is non-specific compared to the specific binding of dehydrogenases and phosphatases via the dinucleotide fold [62, 63, 64, 65]. The discovery of the binding of proteins to cibacron blue was serendipitous [66, 67]. More recent work in this area has used immunoaffinity columns for the removal of albumin [68, 69, 70, 71]. Phage display has been used to develop a specific peptide sequence for binding to albumin and subsequent removal from serum [72].

Removal of IgG is accomplished with either Protein A column or Protein G columns [69, 70, 71, 73]. As a final note, these approaches are all based on "negative selection affinity" where "contaminating proteins" are removed and without analysis for the removal of other proteins, the process cannot necessarily be considered to be a validated process in preparing samples for subsequent analysis. Davidsson and coworkers [74] have compared several depletion matrices for effectiveness in removing high

abundance plasma/serum proteins (albumin, IgG, α_1-antitrypsin, IgA, transferrin, haptoglobin). Affinity matrices included reactive dyes, protein A, polyclonal antibodies, anad protein G. Techniques include liquid chromatography columns, gravity-flow columns, and centrifugal column (spin-tubes). These investigators concluded that the use of multiple polyclonal antibodies was the most promising approach. However, again there was no attempt to identify low-abundance species which may have been "lost" in the process. There have been other approaches suggested for the prefractionation of serum. Zhang, Bast and coworkers [75] used anion-exchange chromatography for the prefractionation of serum prior to surface-enhanced laser desorption/ionization mass spectrometry (SELDI) [76]. Specifically, serum was applied to an anion-exchange column (matrix not defined) and discrete fractions were obtained with an isocratic pH gradient. Portions of each fraction were taken to a variety of ProteinChip® matrices including SAX (strong anion exchange), (IMAC3-Cu) Copper ion IMAC, H50 (hydrophobic), and WCX2 (weak cation exchange). Pieper and coworkers used "multifunctional" immunoaffinity (Imummoaffinity subtraction chromatography, ISAC) [77]. Polyclonal antibodies to a variety of plasma proteins were coupled to a matrix by binding to immobilized protein G (for goat antibodies) or protein A (for rabbit antibodies) and the complexes stabilized by covalent crosslinking with dimethysuberimidiate. A variety of columns were evaluated for ability to remove high-abundance plasma/serum proteins such as albumin, immunoglobulin G (IgG), α_1-antitrypsin, haptoglobin, immunoglobulin A (IgA), and α_2-macroglobulin. Several antibody resins were combined to prepare a "multifunctional" column. For example, an IASC resin could be composed of immobilized antibodies to albumin, transferrin, haptoglobin, α_1-antitrypsin, α_1-acid glycoprotein, α_2-HS glycoprotein, hemopexin, transerythrin, and antithrombin with half of the matrix being an anti-albumin matrix. This approach is more efficient that the use of a multiple immunoaffinity columns and does yield a cleaner sample for two-dimensional gel electrophoresis. While this is an improvement over less specific approaches such as immobilized dyes, the studies still do not address the issue of the removal of the "unknown protein." Zhang and coworkers [78] used a combination of multidimensional liquid chromatography and gel electrophoresis with mass spectrometric (MALDI/Q-TOF; ion trap liquid chromatography-tandem mass spectrometry) to study plasma protein biomarkers in Alzheimer's Disease, type-2 diabetes,

and congestive heart failure. Liquid chromatography matrices include anion-exchange (quaternary amine, DEAE), cation-exchange, lectin-based affinity matrices (concavalin A), IMAC, and heparin. Samples were either analyzed by SELDI technology and/or by one-dimensional or two-dimensional electrophoresis followed by tryptic digestion/mass fingerprinting. Finally Fountoulakis and coworkers [79] have evaluated several different methods for the precipitation of plasma/serum proteins prior to proteomic analysis. These investigators found that ultrafiltration, precipitation with trichloroacetic acid or acetone was useful for the preparation of samples. They also observed that ammonium sulfate precipitation could be useful as it could separate albumin; however, other proteins are also likely removed.

CONCLUSION

While each biomarker search will provide it's own challenge, the following recommendations are presented as a starting point:

- A proactive protocol for sample processing should be developed and used in the study. Samples should be obtained at the same time to avoid possible complications from diurnal or circadian variation.

- Serum is preferred to plasma and must be collected into a glass container placed into storage within an uniform period of time; one hour at $25 \pm C$ is recommended. A stability study is recommended after initial studies.

- If plasma is used, EDTA is the recommended as the anticoagulant and an absolutely uniform process established for obtaining plasma.

- In the absence of other information, storage of samples at $-80 \pm C$ is recommended; samples must not be used more than one. It is possible that a sample could be stored as a reference sample but not as a primary sample.

- Normal values for potential biomarkers must be established prior to the experimental series; it is recommended that 30 normal samples be used. The individual investigator will need to establish whether normal samples need to be age-matched, gender-matched, etc.

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