

Significance of Serum Samples for Protein Studies

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Abstract:

Serum proteins also known as blood or plasma proteins are proteins present in blood that serve many different functions, including transport of lipids, hormones, vitamins, and minerals in the circulatory system and the regulation of acellular activity and functioning of the immune system. Other blood proteins act as enzymes, complement components, protease inhibitors, or kinin precursors. Although serum proteins have very high concentrations, they exhibit an uneven distribution in terms of composition. That is, only about 22 proteins account for 99% of all the serum proteins. These include serum albumin, globulins, and fibrinogen. The remainder 1% of blood proteins is composed of low abundance circulatory proteins, as well as proteins secreted by live, apoptotic, and necrotic cells. Most blood proteins are secreted by the liver and intestines except for the gamma globulins, which are synthesized by the immune system. In this study we aimed to analyse the serum samples taken from gastric cancer patients and compared with healthy serum samples. We estimated the serum samples using Bradford method of protein estimation and performed SDS PAGE analysis. This article highlights the importance of serum sample collection, handling and storage with respect to consideration towards protein-based studies using serum samples.

Keywords: *Serum, Protein Estimation, SDS PAGE, Gastric Cancer*

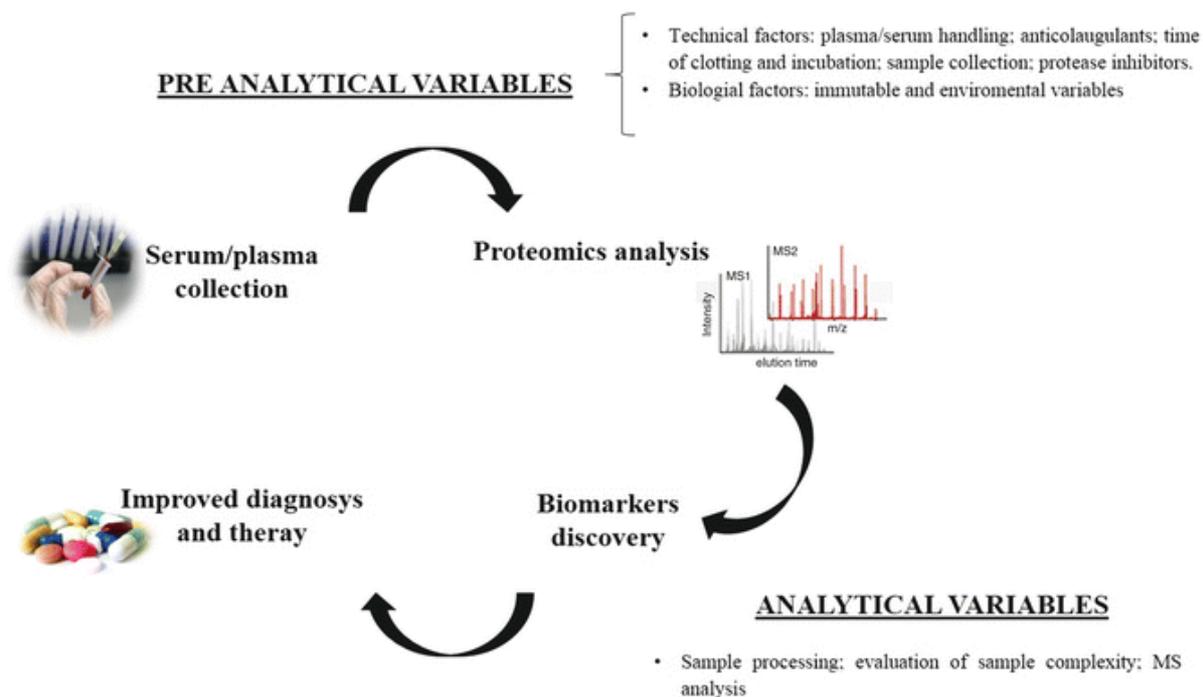
Introduction

The human serum is composed of circulating carrier of exogenous and endogenous liquids in the blood. Human serum thus helps in the movement and transportation of fatty acids and thyroid hormones, which act on most of the cells found in the body. Plasma & Serum, a valuable source for various proteome analysis because of their availability and stability for the discovery of biomarkers & for the study of the outcome of human pathologies (Geyer PE *et al.*, 2016; Pieragostino D *et al.*, 2010). All body cells communicate through blood. For this specific reason, serum & plasma is considered to be a key source of physiological information about the complete status of each tissue, and for several pathological conditions.

With respect to human proteome, the blood proteome is one of the most complex component in them. Its protein content reflects both the physiological and pathological status of each individual. Simple sample withdrawal is one of the most important advantages for blood samples. Urine or saliva are easier to collect, but they are less informative and stable and show higher variability according to the time of collection and individual habits.

Blood is composed of different components which includes lipids, salts, amino acids, carbohydrates, and a large sort of proteins. It collects proteins from tissues and organs from the whole body and contains more than 10,000 different protein classes (Thadikaran L *et al.*, 2005; Adkins JN *et al.*, 2002). These numbers, however, just partially reflect the real complexity of protein species, which is determined by the huge number of different proteoforms and classes of antibodies (Liotta LA *et al.*, 2003). Serum or plasma which is composed of 90% of high-abundant proteins (HAPs).

Figure 1: Schematic Representation of Biomarker Discovery



Albumin alone takes into 50% of the total protein and fibrinogen and haptoglobin contributed to the remaining 40% of whole plasma proteome (Lista S *et al.*, 2013). The complexity of serum/plasma proteome likely to be increased by presence of these bigger proteins and by a large number of posttranslational modifications (PTMs) (Anderson NL and Anderson NG, 2002). HAPs are very often not considered valuable as putative biomarkers (Hu S *et al.*, 2006); however, their relative abundance and their cleaved or modified forms could precisely reflect physiological and pathological status (Hortin GL and Sviridov D, 2010). In this study we have performed the protein estimation from the serum samples and analysed the serum protein samples using SDS PAGE.

Materials and Methods

Study population and Sample collection

All the study subjects included in this study were of South Indian Tamil origin and the ethical approval was granted by the Institutional Ethical committee of Madras Medical College (MMC), Chennai (EC Reg No. ECR/270/Inst./TN/2013; IEC No. 31082015). Patients with primary GC (n=6) admitted in the Dept of Medical gastroenterology, MMC Chennai, were provided with written informed consent and their blood samples were collected from them during the period December 2018 to October 2019. A self-proclaimed healthy individual (n=6) who are free from gastrointestinal diseases and other cancers were recruited as control for the study. Serum from the whole blood sample was separated by centrifuging at 3500 rpm for 8 min. Later, the supernatant was collected and stored at -80°C until further processing.

Results and Discussion

Serum sample preparation & SDS page analysis

Table 1 describes the sample details. Table 2 describes the demographics of the subjects used in the study.

Table 1: Clinical Characteristics of Patient samples

Sample ID	Age/ Sex	Malignant Status
1	64/ M	Healthy
2	59/ M	Healthy
3	56/ M	Healthy
4	60/ M	Healthy
5	58/ M	Healthy
6	61/ M	Healthy
7	63/ M	Gastric Cancer
8	57/ M	Gastric Cancer
9	60/ M	Gastric Cancer
10	60/ M	Gastric Cancer
11	63/ M	Gastric Cancer
12	63/ M	Gastric Cancer

Healthy cohorts of South Indian Tamil ethnicity (n=6) who were willing to participate in the study and devoid of gastrointestinal complications, diabetes mellitus, autoimmune diseases were included. Gastric cancer patients (n=6) were enrolled for the study.

Table 2: Demographic of the Subjects used for study

		Healthy control(n=6)	Gastric cancer(n=5)
Age(yrs)	Mean \pm S.D	60 \pm 2.7	61 \pm 2.4
Gender	Male	6	5

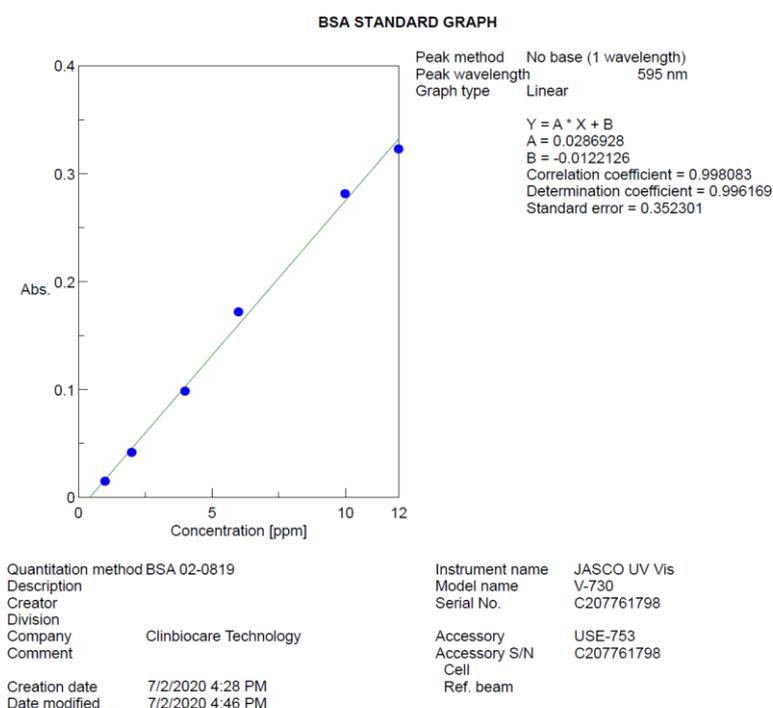
Protein estimation

For estimating the protein concentration of serum samples, the standard graph has to be made. Bovine Serum Albumin (BSA) was used as a standard protein to generate the standard graph which will further help us in identifying the concentration of serum samples. Known concentration of the standards were made from working stock concentration of BSA of 1 mg/ml. Totally seven standards were made, the range of the concentration is from 1 ug, 2 ug, 4 ug, 8 ug, 10 ug, 12 ug. After BSA standard preparation, corresponding volume for all the standards were added separately into the tubes and made up to 1000 ul using milli q water as described in table 3.

Table 3: Details for preparing the standards

Std Number	Volume of BSA working concentration (1 mg/ml)	Volume of Milli Q water	Known concentration of BSA in ug	Absorbance OD at 595 nm
STD 1	1 ul	999 ul	1 ug	0.015
STD 2	2 ul	998 ul	2 ug	0.0416
STD 3	4 ul	996 ul	4 ug	0.0984
STD 4	6 ul	994 ul	6 ug	0.1719
STD 5	8 ul	992 ul	8 ug	0.2577
STD 6	10 ul	990 ul	10 ug	0.2813
STD 7	12 ul	988 ul	12 ug	0.3227
BLANK	-	1000 ul	-	0.000

After preparing the standards, 1000 ul of Bradford reagent (Sigma Aldrich, Germany) was added to the standards and incubated at room temperature under dark condition for 10 minutes. After incubation period, the samples were analysed in Jasco (Maryland, USA) UV – Dual Beam Visible Spectrophotometer Model V - 730 at 595 nm. The analytical program namely Spectra Manager II was used to take the readings and the standard graph was generated and showed in figure 2.

Figure 2: Standard graph with BSA for protein estimation

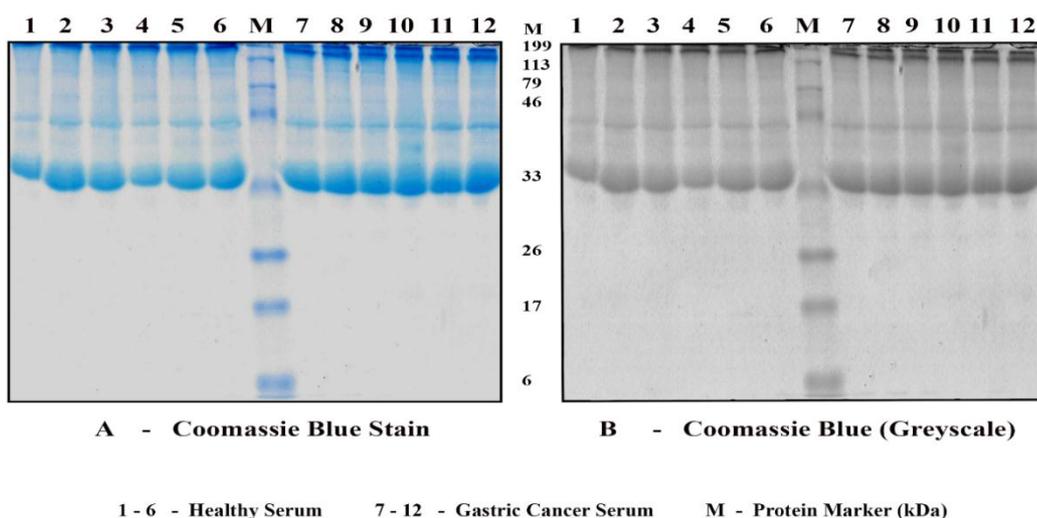
By applying this standard graph, the unknown serum samples were estimated and the concentration of the protein was detailed in table 4.

Table 4: Protein estimation for the serum samples

Sample ID	Absorbance OD at 595 nm	Concentration of Protein in ug/ul
1	1.52	52.75
2	1.53	52.94
3	1.50	51.88
4	1.43	49.48
5	1.52	52.52
6	1.65	57.00
7	1.54	53.11
8	1.62	55.87
9	1.59	54.83
10	1.49	51.39
11	1.56	53.80
12	1.63	56.21

SDS PAGE Analysis

The estimated proteins were further analysed by SDS PAGE analysis to check the quality and quantity of the protein from the serum sample. Figure 3 shows the protein pattern of the serum samples obtained from Gastric cancer patients and healthy subjects. The lane number 1 to 6 represents six samples of healthy subjects and the lane number 7 to 12 represents six samples of Gastric cancer patients. The protein marker is loaded to understand about the protein expression patterns with respect to molecular weight. The gels were stained with CBB 250 stain to visualize the protein bands.

Figure 3: SDS PAGE analysis for serum samples

Sample Selection and Collection

It is a deception that plasma and serum are a similar liquid. As per a clinical science perspective, serum varies from plasma simply because it lacks fibrinogen, however they generally vary for protein composition and concentration. Protein and peptide profiles of plasma and serum have been reported to be dissimilar (Tammen H *et al.*, 2005; Banks RE *et al.*, 2005).

Modes of sample withdrawal

Sample collection mode represents a critical step for two aspects, one related to patient and withdrawal and the other one linked to laboratory practice (Lippi G *et al.*, 2006; Lippi G *et al.*, 2005). The patient's posture (if he is standing, lying, or sitting), the tourniquet application, the site of withdrawal, and the use of alcohol to clean the skin can cause haemolysis and influence proteomics analysis (Lippi G *et al.*, 2006 & 2005). To avoid the concentration of CSF biomarkers, blood drawl was done toward the beginning of the day, prior to 10 a.m fasting (Rodriguez AD and Gonzalez PA, 2009). In the step of drawl, a 21-G needle is preferred to avoid the risk of haemolysis. The middle cubital vein, as a rule effortlessly found and got to, is viewed as the favoured site. The skin should be washed with alcohol to evaporate in order to avoid the haemolysis consequently to blood contamination (Rodriguez AD and Gonzalez PA, 2009).

Anticoagulants

The handling of plasma devoid of clotting is clearly laborious; nonetheless, some different precautionary measures are mentioned. In a biomarker study, the decision for serum or plasma and, if plasma, for which anticoagulants to utilize, speaks to a significant advance to address appropriate sample preparation. Plasma can be naturally converted to serum at RT by the action of the proteases of thrombin on fibrinogen and on the other components of the coagulation cascade (Lista S *et al.*, 2013; Capila I and Linhardt RJ, 2002; Dammann CE *et al.*, 2006). The use of anticoagulant (EDTA, heparin, citrate) is requested for plasma separation in order to protect plasma samples from clotting (Lista S *et al.*, 2013).

The Human Proteome Organization (HUPO) highlighted that there is the need of standardized guidelines for each anticoagulant (Rai AJ *et al.*, 2005). For a quantitative proteomics study, EDTA is by all accounts the elective anticoagulant (Ahn S-M and Simpson RJ, 2007). However, in general, it is necessary to evaluate the single type of experiment, because the use of anticoagulants may affect the stability of some specific proteins (Jambunathan K and Galande AK, 2014). Truth be told, the anticoagulant can likewise introduce a few disadvantages (Luque-Garcia JL and Neubert TA, 2007). For example, the heparinized samples seem to be more stable (Dammann CE *et al.*, 2006); therefore, heparin can interact not only with antithrombin III factor but also with a considerable number of different proteins (Capila I and Linhardt RJ, 2002). Moreover, being a highly charged molecule, it can influence the binding of other molecules in solution, and it is important in some particular techniques such as chromatographic separation (Lista S *et al.*, 2013, Dammann CE *et al.*, 2006). EDTA, an anticoagulant with negative charge, can bind to metal ions and it decreases the reactivity. So it is not specified to use EDTA in the tests through the use of divalent cations. EDTA seems less stable in comparison to heparin (Banks RE *et al.*, 2005), but it represents the right strategy to inhibit proteases that necessitate metal ions for the (Ahn S-M and Simpson RJ, 2007) coagulation process (Banks RE *et al.*, 2005). Another

negative aspect depends on EDTA's ability to induce platelet aggregation altering the plasma proteome content (White JG, 2000). The other anticoagulant Citrate can able to bind calcium leading to a delusional effect after the addition of blood during sample preparation (Rai AJ *et al.*, 2005). In this event, it is very critical to calculate the correct ratio of anticoagulant/ blood in order to not dilute extremely the sample (Rai AJ *et al.*, 2005).

Protease inhibitors

To avoid the degradation of protein, protein inhibitors sought to be added to the samples. HUPO HPP emphasized the significance of the protease inhibitors starting to the sample collection phase. By the analysis of various peptide peaks obtained by SELDI-TOF-MS, it's been demonstrated that plasma proteomics profiling of samples treated with inhibitors is more stable than other untreated (Rai AJ *et al.*, 2005). In general, both serum & plasma citrate where witnessed to be most proteolytically active trailed by plasma heparin & plasma EDTA. Indeed, these molecules can alter active sites of proteases like trypsin and chymotrypsin. Furthermore, they can alter serine residues on other proteins by adding an amine group, thus shifting the pI to higher values. All these modifications can be evaluated by smearing 2D gel (Lista S *et al.*, 2013).

Collection tubes

The other factors can modify the content of proteome during sample collection. Sometimes, the covering materials such as Silicones, surfactants which has polymeric material, plastic covering and polymeric gels adapt to take extra care of viscosity, are often released from tubes, and these might change the content of serum & plasma, these will be disturbing along with spectrums of sample which are detectable with MALDI spectra (Drake SK *et al.*, 2004). A specific study was made to evaluate the effect of using different types of tubes in altering the protein profile, it was found that the protein profiling gets different based on the tube, in which they are collected (Hsieh SY *et al.*, 2006; Villanueva J *et al.*, 2005). Collection tubes preloaded with a protease inhibitor cocktail and anticoagulants have been found to produce reproducible plasma samples (Percy AJ *et al.*, 2013). By contrast, there is no serum collection tubes have yet been developed commercially which can reproducibly produce serum samples for proteomics studies. For this reason, HUPO has recommended using plasma instead of serum, and this is the rationale for other mass spectrometry group (Percy AJ *et al.*, 2013; Omenn GS, 2004; Omenn GS, 2007; Omenn GS *et al.*, 2005).

Sample Stability and storage

A recent study observed that the proteins from plasma are more stable even after multiple freezing and thawing (Zimmerman LJ *et al.*, 2012). The same data was confirmed by a recent study done by Mateos *et al.* (Mateos J *et al.*, 2017). Even though the major stability, numerous studies done by Martino *et al.* (Martino TA *et al.*, 2007), and Mann (Robles MS and Mann M 2013) show a kind of diurnal variations of the concentrations of some proteins as plasminogen, transthyretin, and apolipoprotein which is involved in few pathways with a circadian response. For this reason, it would be better to collect the sample in the same day (Lundblad RL, 2003).

Serum proteome differences are strictly linked to the time of coagulation and temperature of storage (Hsieh SY *et al.*, 2006). Apweiler *et al.* demonstrated that serum peptide profiling could be modified during clotting by the time (30–60 min) (Apweiler R *et al.*, 2009). Experiments were performed to find the efficacy of storing samples in ice and it

was found that serum proteins were changes a lot when kept at room temperature for one hour whereas no major changes observed when it is stored in ice for the same time. As recommended by HUPO, serum should be obtained 60 min after clotting at RT and subsequently stored at 4–8 °C (Apweiler R *et al.*, 2009) or, preferably, at –80 °C before analysis (Apweiler R *et al.*, 2009).

Temperature

However, as highlighted also for other fluids (Greco V *et al.*, 2014; Rosenling T *et al.*, 2011; Pieragostino D *et al.*, 2013), the temperature is the key element for the maintaining the stability of proteome and its functional enzymatic activity, hence proper temperature should be maintained from sample withdrawal to storage of the samples. Several studies have been performed concerning these critical factors (Marshall J *et al.*, 2003; West-Nielsen M *et al.*, 2005).

A recent study described (Rai AJ *et al.*, 2005) that storage with liquid nitrogen would represent the ideal condition to guarantee protein stability, and when it is not possible, temperature should be set at –80 °C, immediately after the handling, preferable in small aliquots (Rai AJ *et al.*, 2005; Marshall J *et al.*, 2003). During sample transfer, proper care should be taken. One should avoid recurrent cycles of storage and will be limiting to 2 refreezing steps (Rai AJ *et al.*, 2005).

Thus all these points to be considered when serum samples is chosen for proteomic studies and each step has its own impact with sample integrity maintenance and stability.

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