Direct Albumin quantification by NanoDrop and Optical Properties of blood plasma

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Abstract: *Human Serum Albumin (HSA) is the most prominent and abundant protein in blood plasma. Deficiency of HSA may cause hypoalbuminemia which is an indicator of cancer. In this study HSA has been quantified in blood samples from nine different cancer patients before (pre) and after (post) first dose of applied chemotherapy drug. Albumin has been quantified by the auto-ranging path length capability of NanoDrop™ 2000. Optical properties of these samples have also been measured. Nonlinear optical properties of human serum albumin from the same human blood plasma has been measured by the Z-scan technique. Change in albumin concentration in the blood serum of a cancer patient undergoing chemotherapy with the corresponding change in nonlinear optical (NLO) response has been calculated. Data analysis reveals a regular change in albumin concentration and a substantial change in the NLO response due to the application of the chemo-drug.*
Key Words: *NanoDrop™ 2000, HSA, Nuclease Free Water, PBS, 0.9% NaCl, Z-Scan, Plasma, Chemotherapy, Nonlinear Optical Response (NLOR)*

I. Introduction

The survival of cancer patients depends on their nutritional status. The serum albumin level is a commonly used method for assessing the nutritional status of cancer patients[1]. These albumin levels could be affected by multiple factors such as infection, injury etc., resulting in malnutrition such as hypoalbuminemia which is a common scenario of most types of cancer. Although serum albumin is a nutritional factor, hypoalbuminemia acts as an inflammatory marker among colorectal cancer patients[2]. Previous studies have shown that the serum albumin levels in chemotherapy are changed in cancer patients[3]. Patients with rich nutritional status have high albumin content and so, chemotherapy induced toxicity might not have been observed in them. But patients with hypoalbuminemia show the opposite results[4].

HSA is a circulating storage and transport medium for many endogenous compounds like several fatty acids, hemin, bilirubin, and tryptophan[5, 6]. In addition to these HSA also carries exogenous drug molecules[7], heavy metal ions because of its versatility in binding capability by means of conformational changes[8-9]. It is the most abundant type of serum protein constituting about 60% of its kind in blood serum that helps vertebrate body to maintain fluid balance[10]. HSA exists in monomeric form and is soluble in water. It is synthesized in liver cells called hepatocytes and is transferred to systemic circulation through hepatic plasma where it has a half-life of about 20 days in humans[11- 12.].

Albumin helps to prevent the fluid from leaking out of blood vessels and supplies nutrients to different parts of the body which helps growth and repair of tissues. There is a strong correlation between HSA concentration (Cp) and mortality rate[13]. Standard clinical diagnosis of health conditions include observation of Cp in blood to look for an increase (hyperalbuminemia) or decrease (hypoalbuminemia) of the value, which are associated with hepatic atrophy, heart failure, dehydration, burns, etc.[14]. Observations show patients, having serum albumin concentrations lower than 2.5 g/dL, exhibit increased drug sensitivity due to higher amounts of the unbound exogenous drug carried by the albumin[15].

Currently established methods of HSA quantitation involve spectrophotometric and spectrofluorimetric assays[16-20]. Recent spectrofluorimetric methods utilize the resonance light scattering (RLS) technique where light in the near UV band scattered by the particles gives information of their native state[21-24]. NanoDrop2000[TM] is a micro volume spectrophotometer developed by Thermo Fisher Scientific for quantitation of nucleic acid, purified proteins, etc. with a high degree of accuracy and reproducibility. The Protein A280 method is applied here to determine concentrations of proteins that contain Tryptophan (Trp), Tyrosine (Tyr), Phenylalanine (Phe)

and cysteine disulfide bonds which all absorb at 280 nm. The linear CCD array makes the measurement possible within seconds[25].

On the other hand, the Sheikh-Bahae formalism of the single beam Z-scan technique has been widely used to investigate third order optical nonlinearity of materials in solution [26, 27]. Biomolecules which in most cases constitute non-conjugated €- bonds are good candidates of optical nonlinearity. This technique is based upon monitoring the transmission of a focused Gaussian laser beam through a sample as it is translated along the beam axis through the focal point. Scan range required depends on the beam parameters and the sample thickness. In Z-scan experiments the sample is made to experience a high intensity laser beam of Gaussian profile, under the action of which the optical properties of the materials alter from their respective values in the linear optical regime and start depending on the incident light intensity, i.e. the incident optical electric field. The order of the nonlinear response largely depends on the molecular makeup of the interacting system and its concentration. Liquid samples which are inherently centrosymmetric in their ordering of constituent particles, exhibit only third order nonlinearity in the form of nonlinear absorption (NLA) and nonlinear refraction (NLR). In the case of a CW laser Z-scan we can look into the nonlinear effect in the thermal regime only where the nonlinearity arises from a change of the refractive index of the medium as a result of radial variation of heating of the medium. The radial dependence of the intensity profile of a Gaussian beam causes position dependent variation in heating and refraction resulting in thermal lensing by the medium. During the passage of a high intensity laser light through the medium the incident region behaves either like a positive or a negative lens in which the incident beam undergoes either self-focusing or self-defocusing respectively. This lensing effect can be effectively studied by the Z-scan method developed by Sheikh-Bahae et.al. in 1989[26] . As the sample under study is made to move along a pre-focused laser-beam axis, the material experiences a variation in incident intensity both along the axis and the radial direction. The effect of this variation is demonstrated in the power transmitted through an aperture placed at a far field position. The normalized transmittance through the aperture as a function of the sample position with respect to the focal plane is either a peak followed by valley in case of negative lensing or a valley followed by peak in case of positive lensing as shown in Fig. [1]. The peak valley separation T_{pv} for a normalized curve is a measure of the on-axis phase shift ($\otimes \sqrt{}_0$) according to the following equation.

Where 'S' is the linear transmittance of the aperture. This phase shift again is a measure of the nonlinear refractive index n_2[28] that is a property of the material and its concentration also. This method has already been used in various bioanalytical applications, such as determination of blood sugar content[29], total cholesterol and trygrycerides[30] total protein and albumin assay[31]. There are some reports on characterization of lipids in body fluid[32, 33] study of the nonlinear refraction of vitreous humor in humans and rabbit[34], and determination of nonlinear refractive index of retinal derivatives[35].

II.   Materials and Methods

In this study a Thermo Scientific NanoDrop[TM] 2000 Spectrophotometer has been used to determine the protein quantity in blood plasma by means of UV light absorption at specific wavelengths by applying the Beer-Lambert law. The auto-ranging path length capability of the NanoDrop™ 2000 allows for the quantification of a very broad range of sample concentrations with serial dilution by different solvents such as Nuclease free water (NFW), Phosphate buffer saline (PBS) and 0.9% NaCl solution. For this reason, eighteen blood samples from nine different cancer patients in their two different chemotherapy treatment stages were collected.

Study location: Nonlinear BioOptics Laboratory, Department of Physics, Shahjalal University of Science and Technology, Sylhet-3114 and Invent Technologies Ltd, Banani, Dhaka, Bangladesh

Study Duration: November 2017 to November 2018.
Sample Size: Blood samples of 9 different cancer patients.
Sample Size calculation: The study population was drawn from cancer patients who were being treated in Osmani Medical College and Hospital and who were given chemotherapy treatment. Collected samples are divided into two categories:
a) Pre chemotherapy stage (B), and
b) Post chemotherapy stage (A)

Study methods:

- **Protein quantification by NanoDrop™ 2000 Spectrophotometric technique**

NanoDrop™ 2000 Spectrophotometer is an innovative technology based on UV analysis of a micro volume sample held by surface tension between two optical pedestals with a fiber optic arrangement. 0.5 to 2 µl samples are placed between the optical pedestals which form a liquid column of desired length. Light from a xenon flash lamp transmitted through the sample is analyzed by a linear silicon CCD array which records the

spectrum in the 190 nm to 840 nm range simultaneously with an accuracy of ± 1 nm. At first the baseline is produced by preparing a buffer in which the protein is dissolved. The buffer has the same pH and ionic strength as that of the protein solution. 1.0 μl of the buffer sample is pipetted on to the lower pedestal and the upper one engaged to form a liquid column using the liquids surface tension property, the path length is adjusted by the systems auto ranging capability. The 'Blank' is then clicked from the menu to load the baseline data. Once the 'Blank' run is finished both the pedestals are cleaned with laboratory wipe. The protein sample is then homogenized on a vortex mixture at medium rpm and pipetted on to the pedestal for UV measurement. Clicking 'Measurement' from the menu initiates the run which is finished within a few seconds and the UV spectrum is displayed.

- **Nonlinear optical response by Z-scan technique**

The experimental Z-scan setup shown in Fig.1 consists of a continuous wave (CW) diode laser emitting 665 nm light beam of nearly gaussian profile, neutral density (ND) filter for adjusting incident power on the sample, F_1 - F_2 lens system for correcting beam profile, F_3 lens for focusing the beam onto the sample and A and B are power meters measuring the incident power on the sample and power transmitted through the aperture. As the sample is translated along the beam line through the focus of F_3 it initially experiences an incident converging beam of increasing intensity on approach to the focal plane and a diverging beam with decremented intensity as it moves away from the focal plane.

In our experiment we determined the phase shift parameter for five different cancer patients. Scans were run on 5 samples from five different patients of before (pre) chemotherapy stage and 5 samples from the same patients in after (post) chemotherapy stage. The incident powers on each sample were varied from 60 mW to 100 mW which is detected by the Power meter 'A'. The optical power transmitted throug the aperture is detected by meter 'B' and normalized. The obtained data at each power is then curve fitted to get the T_{pv} [36, 37] which gives the on-axis phase shift ($\otimes\sqrt{0}$). Obtained results are then analyzed and presented in graphical form.
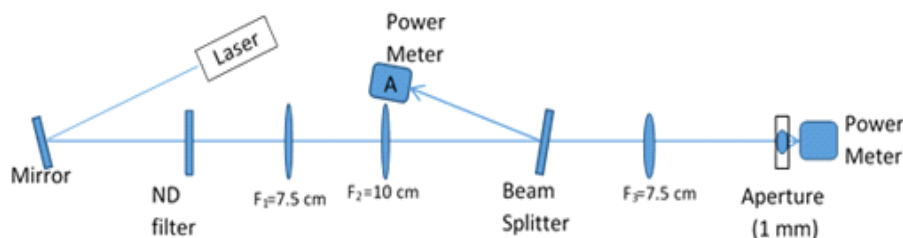

Figure 1: Closed aperture Z-scan setup

Sample preparation:
Sample preparation for albumin quantification by NanoDrop^{TM} 2000 spectrophotometer:

Samples for albumin quantification were prepared using three different solvents Nuclease free water (NFW), Phosphate buffer saline (PBS) and 0.9% NaCl solution. In the first step 50 μL of blood plasma were added to 50 μL of Nuclease free water (NFW) in a micro centrifuge tube and mixed properly by a shaker. In the second step 50 μL of solution from step one was added to 50 μL of Nuclease free water (NFW) in another micro centrifuge tube and mixed properly. In the third step 50 μL of solution from step two was added to 50 μL of Nuclease free water (NFW) in another micro centrifuge tube and mixed properly. In the fourth step 50 μL of solution from step three was added to 50 μL of Nuclease free water (NFW) in another micro centrifuge tube and mixed properly. Same procedure was employed for samples prepared using two other solvents, Phosphate Buffered Saline (PBS) and 0.9% NaCl.

Sample preparation procedure for Z-scan technique:
250 μL of blood plasma and 250 μl of Nuclease free water from milli-Q^{TM} were taken into a 1 ml microcentrifuge tube and mixed properly by a shaker. About 300 μl of the sample was then injected into the specially designed quartz cuvette of 3.0 mm path length which was then placed on the sample holder in the Z-scan setup.

III.   Results and Discussion

The results of protein quantifications done by NanoDrop on eighteen samples of blood plasma from 9 different individuals undergoing chemotherapy at the oncology Department, OMCH both pre and post chemotherapy stages are summarized in the figures 2 to 4.

Figure 2 shows average results of protein quantitation in blood plasma of 9 different cancer patients in both pre (B) and post chemotherapy (A) stages as a function of dilution. The dilution was varied from 50 % to 6.25 % and the protein quantity in the samples are found to vary linearly with the percentage of dilution for all the samples. The consistency of the results in protein concentration measurement becomes evident when we look at the figure 3 which shows the results of protein concentration measurement at 50 % dilution in three different solvents like Nuclease free water (NFW), Phosphate buffer saline (PBS) and 0.9% NaCl solution. Here the identity of patients and concentrations of proteins are shown along the X and Y axes respectively. A particular color represents the protein quantity measured in a particular solvent either pre or post chemotherapy. Blue, dark red and green colors represent the protein quantity measured in NFW, PBS and 0.9% NaCl solution respectively. The patients are numbered from 01 to 9 with B suffix referring to pre chemo stage and A suffix post chemo stage respectively. The results are very consistent with very small value in their standard deviation. So any of these three solvent is suitable as a diluent in protein dissolution for experimental purposes.

Now the average of protein concentration measured in the three different diluents at 50% dilution is presented in figure 4. Here the protein content in blood plasma of 9 different patients from pre chemo stage (B) to post chemo stage (A) is presented side by side. Except for two cases of patient 03 and patient 06 there are no significant changes in the blood plasma protein contents. These two patients showed slight increases in their plasma protein content indicating improvement of their health condition.

Figure 2 shows the results of protein content measurement in blood plasma of all the studied samples at four different levels of dilution. The variations of protein content among different samples at these dilution levels appear consistent. According to the results depicted in this figure these three solvents NFW, PBS and 0.9% NaCl solution are suitable solvents for dissolution of plasma protein in the dilution range of 6.25 % to 50 %.
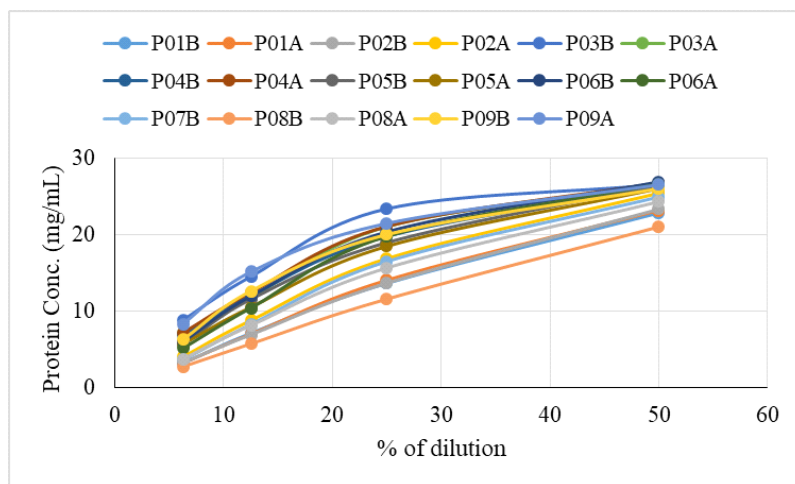

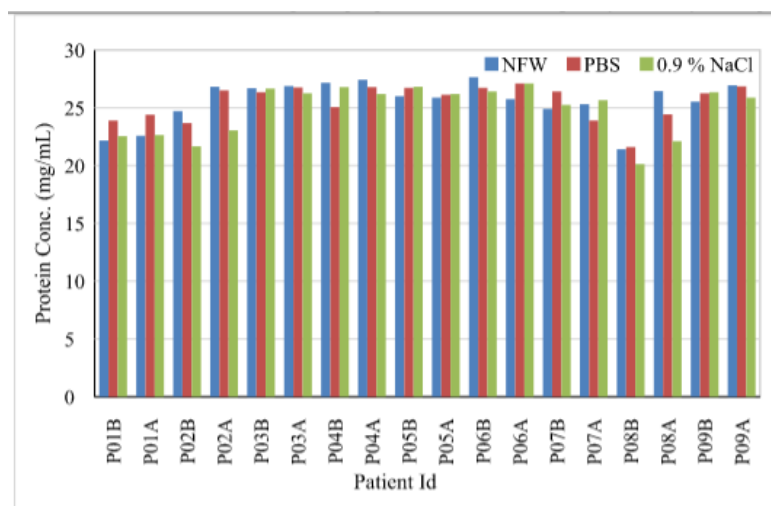**Figure 2:** Protein quantitation at different dilution

Figure 3: Protein quantity measured in NFW, PBS and 0.9 % NaCl diluted blood plasma both pre and post chemotherapy
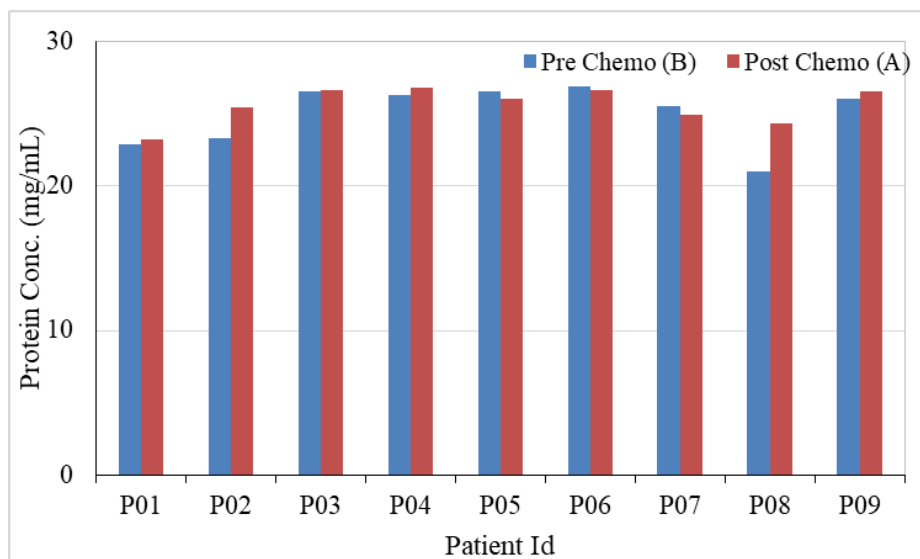
Figure 4: Average of Protein concentration measured at 50 % dilution
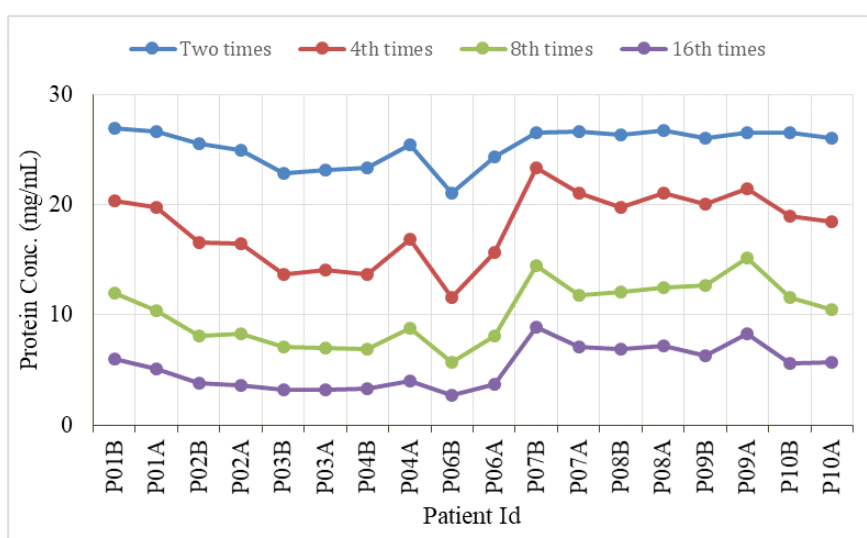

Figure 5: Average of protein quantity measured in NFW, PBS and 0.9 % NaCl diluted blood plasma of patients undergoing chemotherapy

Figure 6 exhibits the NLO response of blood plasma of patient P01 pre chemotherapy stage at different incident laser powers of 60 mW to 100 mW. The curves are the normalized transmittance through a distant aperture as a function of the sample cuvette position with respect to the beam waist position. With the increase of power, the peak valley distance Tpv increased revealing the increase of nonlinear phase shift with power.

Figure 7 shows the comparison between the Z-scan curves of sample P01 at 80 mW incident power at pre chemotherapy and post chemotherapy stages. The Tpv of the post chemotherapy curve is larger than the pre chemotherapy curve, revealing an increase in nonlinearity in samples in post chemo stage. The albumin concentration of this sample has been found to increase as indicated by the average result of three measurements and hence the increment in nonlinearity can be attributed to the albumin content of the samples.

The on axis nonlinear phase shifts at powers from 60 mW to 100 mW for samples P01, P02, P03, P04 and P05 were determined for both pre and post chemo stages and the results are summarized in figure 8. Though the distributions are highly scattered, there is a general trend in increase in the phase shift with power, for all the cases. The nonlinear phase shift values for most of the post chemo samples are greater than their respective pre chemo values. The average of the phase-shifts for a particular sample is calculated and results of five patient samples at pre and post chemo stages are summarized in figure 9. Out of the five samples only P03's nonlinearity decreased in the post chemo stage, whereas the rest of the samples' third order optical nonlinearity increased as reflected in their increase in phase-shifts. These changes in NLOR can be attributed to change in albumin content of blood plasma and also to the effect of administering the chemo drug on the individual's plasma sample.
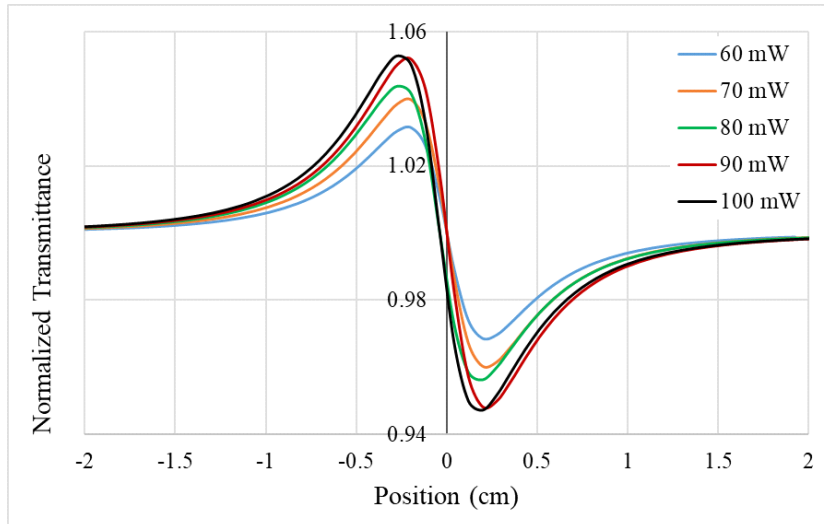
Figure 6: Curve fitted Z-scan curve for Sample P01pre chemotherapy at different powers
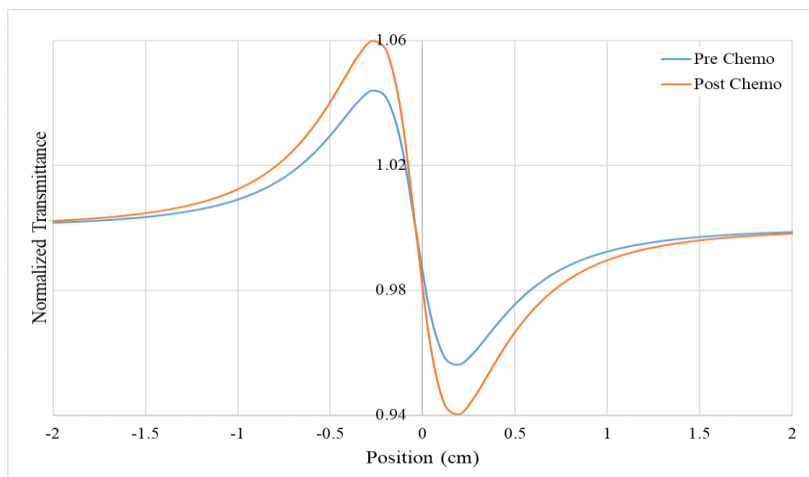

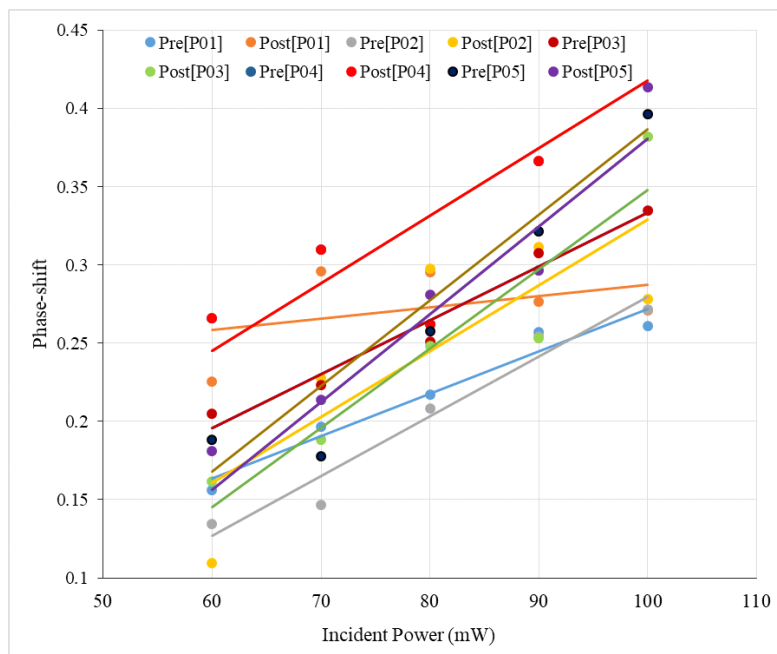**Figure 7:** Curve fitted Z-scan curve for sample P01 at 80 mW power both pre and post chemotherapy

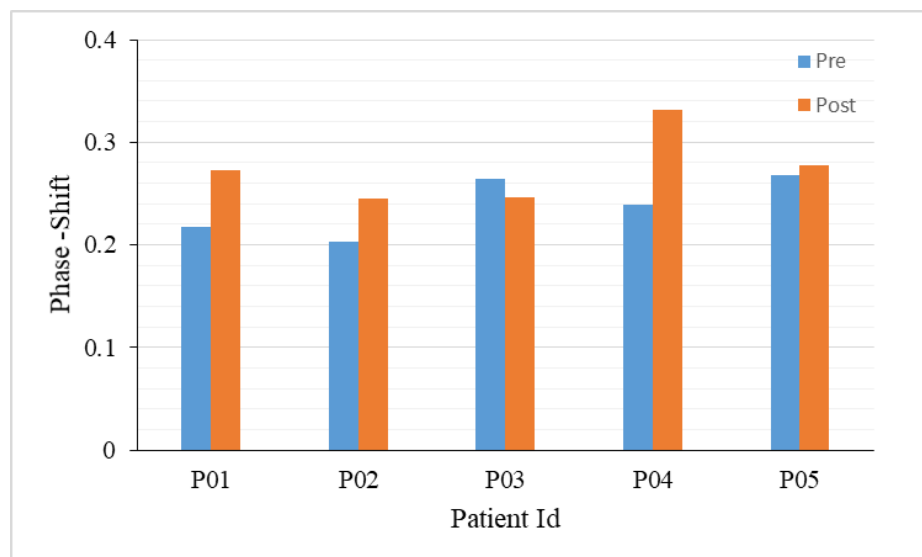**Figure 8:** Nonlinear on-axis phase shift variation with incident power

Figure 9: Average on axis nonlinear phase-shift

IV. Conclusions

Since the protein quantities in the samples are found to vary linearly with the percentage of dilution for all the solvents, the results are very consistent with a very small value in their standard deviation. So any of these three solvents is suitable as a diluent in protein dissolution for experimental purposes. Again out of five samples that were studied for optical nonlinearity, the third order nonlinearity in post chemo stage have increased for four and decreased for one. Two of these four samples have increased albumin content and two have decreased. This third order nonlinearity blood plasma may be due to plasma protein and other factors such as circulating cell free DNA. The increase in nonlinearity in the post chemo stage may be attributed to the effect of chemo drugs.

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