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Short Communication

Separation of Lipoprotein Fractions by Selective Precipitation Method for Carotenoid Analysis

Bagmita Bhagawati*[1] and Bhabesh Chandra Goswami[2]

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Carotenoids are tetra-terpenoid C_{40} compounds that are present in plants, algae and photosynthetic bacteria. Since carotenoids cannot be synthesized in the body of human beings, so the only source of carotenoids is exclusively from the diet. Carotenoids play a diverge role in the human beings ranging from the metabolic conversion of hydrocarbon carotenoids to vitamin A to other activities like antioxidant activity, cell communication gap and free radical quencher. Carotenoids are mostly involved in scavenging reactive oxygen species, singlet molecular oxygen and peroxyl radicals. Carotenoids scavenge peroxyl radicals which are generated in the process of lipid oxidation and interrupt the reaction that finally leads to damage in lipophilic compartments.

Oxidation of low-density lipoproteins (LDL) plays an important role in the development of atherosclerosis. During oxidation of LDL, polyunsaturated fatty acids of LDL are oxidized producing a number of oxidation products. These produces foam cells which aggregates and form the fibrous plaque thereby leading to the development of atherosclerosis. High Density Lipoproteins (HDL) cholesterol levels on the other hand are inversely related to the risk of coronary artery diseases. HDL acts by reversing the stimulatory effect of oxidized LDL on monocyte infiltration. The diets with antioxidants including carotenoids are believed to slow the progression of atherosclerosis [1]. Studies carried out by [2] long time back as well as by [3] and [4] reported β-carotene and lycopene rich foods delays the onset of LDL oxidation. A study conducted in Maryland [5] reported that overweight older women or women having lower serum carotenoids are likely to have high lipoprotein oxidation. Further, the authors suggested that reducing weight or increased intake of carotenoid rich foods may reduce lipoprotein oxidation. [6] reported that lycopene modulates HDL functionality towards antiatherogenesis. Many epidemiological studies reported an inverse association between β-carotene concentrations in plasma and atherosclerosis [7-8]. Research conducted by [9] also found that low levels of HDL-cholesterol and β-carotene are associated with coronary atherosclerosis. Plasma β-carotene levels were inversely correlated with the levels of IL-6 in patients with Coronary Artery Diseases (CAD), besides β-carotene xanthophylls like lutein, zeaxanthin also have an association with CAD [10]. Plasma lutein concentration reduces the concentration of oxidized LDL thereby protects against atherosclerosis [11]. Studies carried out in general populations also reported an inverse association between β-carotene and other carotenoids with inflammatory markers like IL-6 or leukocyte count [12-14]. A study [15] carried out in UK with type 2 diabetes patients demonstrated that increase intake of fruits and vegetables increases the carotenoid levels which helps in enhancing the antiatherogenic potential of HDL. Carotenoids are therefore considered as an important compound for its antioxidant capacity in increasing the oxidative resistance of LDL.

The pandemic outbreak of Coronavirus disease (COVID-2019) is a fatal and highly contagious disease. Since there is still relatively limited knowledge about COVID-2019 but evidences suggest that excessive reactive inflammation, oxidation and an exaggerated immune response very likely to contribute to its pathology [16]. The immune system protects against viruses and diseases and produces antibodies to kill pathogens. Study reveals that β-carotene is a powerful antioxidant that can reduce inflammation and boost immune function by increasing disease-fighting cells in the body [17].

Recent studies considered asthaxanthin (red-orange oxycarotenoid pigment) to have potent anti-oxidant and broad-spectrum anti-inflammatory effects bioactive molecule which plays important role in modulating the immune response thereby reducing the complications of COVID-19 [18-19]. Carotenoids being lipophilic follow the same absorptive pathway as other lipids are therefore transported by the lipoproteins. Once the carotenoids reached the liver, they are exported to various tissues by lipoproteins like low density lipoproteins or high-density lipoproteins. Chylomicron carotenoids are high after 4-8 hours ingestion of carotenoids. In the fasting states, most plasma carotenoids are associated with LDL and HDL [20].

In this regard the present study was carried out to estimate major carotenoids i.e., lutein and β-carotene in serum lipoprotein fractions isolated from fasting subjects. In order to estimate the carotenoids in the lipoproteins, firstly the

***Bagmita Bhagawati**
bagmita.chem@gmail.com

[1]Department of Chemistry, Pragjyotish College, Guwahati - 781 009, Assam, India

[2]Vice-Chancellor, Cotton University, Guwahati - 781 001, Assam, India

lipoproteins have to be isolated. Separation of lipoprotein fractions are based on differences in physical properties which include ultracentrifugation, electrophoresis and precipitation method. In a subset of Sixty-three volunteers, carotenoids were analyzed in the serum as well as in lipoprotein fractions after isolating them from serum. The correlation between serum carotenoid i.e., lutein and β-carotene concentration and cholesterol (C), triglycerides (TG), High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL) and Very Low-Density Lipoprotein (VLDL) was also studied.

MATERIALS AND METHODS

All the experiments were carried out in yellow light in order to avoid degradation of the molecules.

Chemicals and solvents

Dextran Sulphate sodium salt (mol wt 9000-20000) was purchased from Sigma Aldrich, USA. $MgCl_2.6H_2O$, NaCl, EDTA, NaOH, dil. HCl were purchased from North East Chemicals Corporation, Guwahati, Assam, India. Solvents used for HPLC were of HPLC grade and purchased from Qualigens Fine Chemicals, Mumbai, India. Solvents used for extraction were of analytical grade.

Study group

Thirty-nine healthy men and twenty-four healthy women (above 25 years of age) are recruited for the study. Full clinical investigation was carried out by consultant Physician in the hospitals that included a physical examination, a routine blood test (including measurement of glucose, cholesterol and triglycerides) and were excluded if they had any significant abnormalities. Moreover, individuals taking vitamin A and carotenoids as supplements or pregnant women were also excluded from the study. Ethical approval for the study was obtained from the Gauhati University.

Collection of blood samples

Blood from the subjects who had fasted for 12-14h were collected and the serum was separated by centrifugation. Lipoprotein separation was then carried out on the same day.

Isolation of lipoproteins from blood serum

Here selective precipitation method [21] was adopted in which High Density Lipoprotein (HDL) was isolated from serum using a solution of dextran sulphate and $MgCl_2$ to precipitate selectively the very low and low density lipoprotein (VLDL and LDL).

Preparation of precipitating reagents

Stock solution of dextran sulphate and $MgCl_2$ was prepared by dissolving 0.5 g dextran sulphate and 5.08 g $MgCl_2.6H_2O$ each in 25ml volumetric flasks. The pH of dextran sulphate solution and Mg^{2+} solution were adjusted to 7 with dil. HCl and dil. NaOH respectively and stored in the freeze. Equal volumes of the above stock solutions were mixed so that the reagent contains 10g/l concentration of dextran sulphate and 500mmol/l of $MgCl_2$.

Precipitation step

Before carrying out the analysis, the serum as well as precipitating reagents was brought down to room temperature. 100µl of the reagent (i.e., combined dextran sulphate-Mg^{2+} working solution) was added to one ml serum. The mixture was then vortexed and allowed to stand at room temperature for 30 mins. This was followed by centrifugation of the mixture for 30 mins. The supernatant containing HDL was then removed by micropipette. The precipitate was dissolved in a solution containing 6.5% NaCl and 0.12% EDTA and kept inside the freeze until further analysis.

Extraction of carotenoids and analysis using HPLC

Carotenoids were extracted from serum as well as from isolated lipoprotein fractions. One ml of serum or lipoprotein fractions was diluted with 1ml water and denatured with 2 ml EtOH. After vortexing the mixture, the extraction was carried out with hexane (containing 0.04% BHT) three times. The hexane layers were pipetted out each time and the pooled hexane extract was evaporated in a rotary evaporator. The residue from hexane extract was redissolved in 100 µl methanol. A 20µl aliquot was used for the HPLC analysis**.** All samples from each volunteer were extracted in duplicate.

The chromatography was carried out using a Shimadzu system composed of two pumps and a photodiode array detector (PDA SPD-M10AVP). The software used was Class M10 A. Reverse phase HPLC was achieved on a supelcosil-LC-8 (25cm x 4.6 mm, 5µm) column with a guard column using a channel at 450 nm. The analysis was carried out by using the solvent system i.e., CH_3CN: H_2O (97.5:2.5) and CH_3CN: DCM (70:30).

Determination of cholesterol, triglycerides, HDL, LDL and VLDL

The concentrations of cholesterol (C), triglycerides (TG) and HDL were measured in a semi-automated chemistry Analyzer (model Humalyzer 3000, make: Human GmbH). Serum concentrations of LDL cholesterol and VLDL cholesterol were calculated from total cholesterol, HDL-cholesterol and triglyceride concentrations using the Friedewald formula [22].

Statistical analysis

The correlation coefficient (r) was calculated between serum lutein and β-carotene concentrations and Cholesterol (C), Triglycerides (TG), HDL, LDL and VLDL. Further, t test was performed to test the significance of r. The percentage of lutein and β-carotene in HDL and LDL + VLDL for each sample was calculated by dividing the concentration of lutein and β-carotene in each fraction by the sum of the concentrations of lutein and β-carotene in HDL and LDL + VLDL.

RESULTS AND DISCUSSION

Mean lutein and β-carotene in the serum, LDL + VLDL and HDL is shown in (Fig 1). The lutein and β-carotene concentrations in serum as well as in the two lipoprotein fractions i.e., LDL+VLDL and HDL. Contrary to the level of lutein, the β-carotene level in the serum of volunteers was surprisingly low. The mean lutein concentration in serum is 15.91µg/dl, 7.83µg/dl in HDL and 6.42µg/dl in LDL + VLDL.

The mean β-carotene concentration in serum is 5.62µg/dl, 3.94µg/dl in LDL+VLDL and 0.913µg/dl in HDL.

The correlation coefficient between serum lutein, β–carotene concentration and cholesterol, triglycerides, HDL, LDL, VLDL was calculated and t test was performed to test its significance. The correlation coefficient along with the calculated t value is given in (Table 1).

Tabulated t value for 61 degrees of freedom at 5% probability level is 2.00. In our study we found that serum lutein and β-carotene is positively correlated with C, HDL and LDL. VLDL and TG had a negative correlation coefficient with serum lutein and β-carotene. Since the calculated t values of serum lutein and C, serum lutein and HDL, serum lutein and LDL, serum β-carotene and HDL are greater than the tabulated t value ($t_{0.05,61}$=2.00). So, the correlation is significant at 5% probability level. We can conclude that there exists a significant positive correlation between these factors.

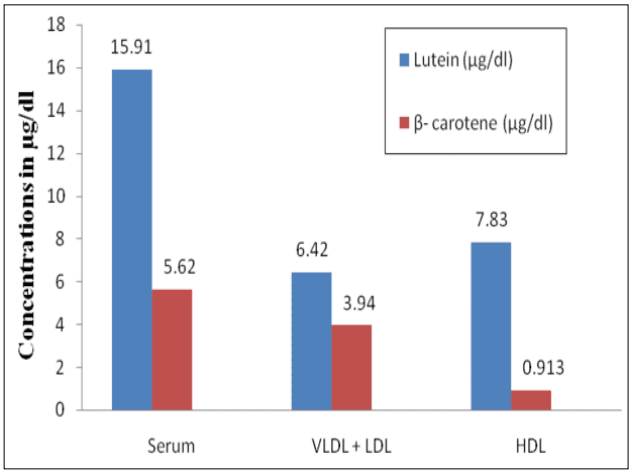

Fig 1 Lutein and β-carotene in serum, VLDL + LDL and HDL

Table 1 Correlation coefficient (r) between cholesterol (C), triglycerides (TG), HDL, LDL, VLDL and serum lutein and β–carotene concentrations

	C	TG	HDL	LDL	VLDL
Serum lutein	0.6751 (3.42)	-0.1392 (0.53)	0.7039 (3.71)	0.5393 (2.39)	-0.1392 (0.53)
Serum β-carotene	0.4564 (1.99)	-0.0815 (0.31)	0.6340 (3.06)	0.4274 (1.77)	-0.0815 (0.31)

Data are presented as correlation coefficient and the calculated t value is reported in parenthesis

The (Table 1) shows the correlation coefficients between serum lutein, β-carotene concentrations and lipid parameters. In our study we observed that the correlation coefficients between serum lutein and VLDL as well as between serum lutein and triglycerides are exactly same. Similarly, the correlation coefficients between serum β-carotene and VLDL as well as between serum β-carotene and triglycerides are same (Table 1). It indicates that the results for triglycerides are exactly same to those of VLDL, since the VLDL is calculated as triglycerides divided by 5. Serum lutein concentration in our study involved a positive statistically significant correlation coefficient with HDL, LDL and C [23-25].

In the present study there exists a significant positive correlation coefficient between serum β-carotene and HDL. A positive correlation coefficient between serum β-carotene and LDL as well as between serum β-carotene and C is found but are not statistically significant. A study was carried out to investigate the relationships between plasma β-carotene, α-tocopherol and oxidative stress in patients with advanced coronary artery disease [9]. They reported that serum β-carotene correlated only with the total cholesterol and LDL cholesterol which has also been observed in our study but is not statistically significant. Another study in Finland has reported serum β-carotene to have a positive correlation with both HDL and LDL [26]. In the current study the pattern of association between lipoproteins and serum carotenoids is found to be same with the studies carried out by other researchers but as the number of samples size incorporated was small in our case, it is found to be insignificant.

SUMMARY

Carotenoids are plant pigments which play significant role in human beings by acting as antioxidant, free radical quencher apart from its role in converting to vitamin A. In the current study carotenoids are estimated in healthy volunteers by using Reversed Phase High Performance Liquid Chromatography (RPHPLC) and correlation coefficient (r) was calculated between serum lutein and β-carotene concentrations and Cholesterol (C), Triglycerides (TG), High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL) and Very Low-Density Lipoprotein (VLDL). Serum lutein concentration in the current study involved a positive statistically significant correlation coefficient with HDL, LDL and C. This study also gives a significant positive correlation coefficient between serum β-carotene and HDL. A positive correlation coefficient between serum β-carotene and LDL as well as between serum β-carotene and C is found but are not statistically significant.

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