Pakistan Journal of Life and Social Sciences
www.pjlss.edu.pk

Determination of Plasma Levels of β- Carotene in Pakistani Population by a Simple HPLC Method

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ABSTRACT

Most of the methods for determination of vitamins are simultaneous for two or more vitamins, but there is need to develop a simple method for β-carotene when it is to be determined individually. The status of β carotene is important to determine because low serum β carotene level is found to have a greater risk of different types of diseases and needs to be determined in order to measure the consequences of the diseases. The present study was done to investigate β-carotene level in the local Pakistani population. The blood samples were taken in fasting. Total of hundred investigates including 50 males and 50 females were selected for this study. All investigates were adults (above 18), on a normal diet and nobody has taken any antioxidant supplementation. β-carotene was found to be 0.61±0.07 μg/mL in serum of total investigates while concentration of male (0.56±0.04 μg/mL) was less than that of females (0.66±0.06 μg/mL). The method used was simple and efficient in sense of sample preparation and mobile phase composition resulting in shorter retention time, sharp peak and clearer results with a lower limit of detection 2.5μg per mL rather than dL. The chromatographic mode used for antioxidant vitamin β-carotene was isocratic and flow rate was 1mL/min while run time and wavelengths for detection of β-carotene was 7.5 minute and 455 nm respectively.

INTRODUCTION

There are many methods for the determination of antioxidant vitamins from human plasma or serum (Hosotani and Kitagawa, 2003; Zhao et al., 2004; Rajendran et al., 2005; Grzelinska et al., 2007; Lee and Ong, 2009; Thibeault et al., 2009; Kandar et al., 2013). Most of them are simultaneous determination methods for two or more vitamins with complex procedures, but if only β-carotene is to be determined, those methods seem to be long and complex. We described here a short method particular for β-carotene determination because of its relation to the risk of development of different types of diseases (Mittal and Mittal, 2004; Kandar et al., 2013). β-carotene, is a fat soluble carotenoid that can be converted into active vitamin A (retinol). It is believed to be most active among more than 50 carotenoids having antioxidant properties and the strong singlet oxygen quencher. It provides

antioxidant protection to lipid rich tissues (Devasagayam et al., 2004).

It has been observed that carotenes, in addition to their vitamin A activity, are able to inhibit or slow the development of tumors induced by dimethy-lbenzanthracene or UV. Highly reactive species are able to be scavenged by carotenoid pigments (Poppel and Goldbohm, 1995). The relationship between vitamin A and cure of cancer was discovered accidentally, after a long time it was observed that people taking the diet rich in beta carotene have lowered the risk for cancer disease. There are contradictory statements about the relationship between lower incidence of cancer, particularly lung cancer and high blood concentrations of β-carotene, but some studies suggest the importance of it in the treatment of different types of cancers. (Hercberg et al., 1998). β-carotene is an antioxidant vitamin that have photoprotective property, useful to protect against UV induced cancer. It is a good

antioxidant and a best quencher of oxygen radicals. Previously finding show that higher serum β-carotene levels inversely proportional to the risk of breast cancer. Some epidemiologic studies illustrate mixed findings for β-carotene levels (Ching et al., 2002). Low serum β-carotene level is found to have greater risk in various forms of cancer,including leukemia and lymphoma (Mukherjee et al., 2011). There are different methods reported for the determination of β-carotene, but most of them are simultaneous determination methods for multiple lipophilic substances and seems to be complex when only β-carotene is to be determined. There was need to have a simple and rapid method for its determination. Present study was designed to standardize a rapid HPLC based detection method followed by the determination of β-carotene in Pakistani male and female population.

MATERIALS AND METHODS

Fifty healthy female and fifty healthy male volunteers were selected. The physical examination and laboratory tests were conducted for each subject to check their health status. The subject who has any type of abnormalities in his history, physical examination or clinical laboratory investigation (Liver function test, Kidney function test, HIV) was excluded from the study. All subjects were adult (above 18 years), non alcoholics, non-smokers on a normal diet and no one was on any medication. Blood was collected after an overnight fasting. All subjects have not taken any antioxidant supplement for one week. The blood sample was collected from each volunteer in fasting. The study was conducted after approval by the Institutional Ethics Review Committee.

Reagents and chemicals
β-carotene acetonitrile, and ethanol from Merck Germany. Deionized distilled water was obtained (Advanced GS-590, Distillery and CPW-200 Japan) from Central High Tech Lab, University of Agriculture, Faisalabad, Pakistan. Methanol, Tetrahydrofuran and chloroform were purchased from Lab scan analytical sciences (Poland S. A.).

Equipments/instrumentation
The high performance liquid chromatographic (HPLC) system consisted of a Shimadzu CBM-20A/ 20 Alite system controller, SPD-M20A UV-Visible photodiode array detector, LC-10AT pump with FCU-10AL VP flow controller valve, DGU-20A3/DGU-20A5 online degasser, and LC-20AT solvent delivery Module. Separation was achieved at LiChrospher® 100 RP-18e column (250 mM × 4.6 mM, internal diameter, 5 µm, Merck, Germany). The chromatographic data was analyzed and collected by using CSW 32 software. Filtration assembly with cellulose acetate membrane filters (Sartorius, AG. 37070 Geottingen, Germany) of pore size 0.45 µm and 47 mM diameter.

Stock solutions and standards
For preparation of standard curve the stock solution (1000 µg/mL) for β-carotene was prepared in chloroform and further diluted to form standard samples having different concentrations (2.5-100 µg/mL) of β-carotene. Representative chromatogram and standard curve is shown in fig 1 and 2.

Preparation of samples
For analysis of beta carotene 500 µL of plasma was deprotenized with 500 µL of absolute ethanol and was extracted with 600 µL of chloroform. The extract was shaken for 5min before centrifuging. The organic layer was extracted and evaporated to dryness under nitrogen. The dried extracts were dissolved in 500 µL absolute methanol and filtered before injecting into the HPLC system.

Chromatographic conditions
The chromatographic mode used for antioxidant vitamin β-carotene was isocratic and flow rate was 1mL/min while run time and wave lengths for detection of β-carotene was 7.5 minute 455 nm respectively. Composition of mobile phase for beta carotene was 50: 20: 25: 5 Methanol: Acetonitrile: Chloroform: Tetrahydrofuran.

Calibration and linearity
Six concentrations were made for calibration curves. Curves were obtained daily for four days generated using linear regression analysis. Standard concentration ranged from 2.5-100µg/mL are shown in Table 1.

RESULTS AND DISCUSSION

In the presence of many methods for the simultaneous determination of lipophillic substances there was need for simple and rapid method for determination of β-carotene concentration when we are solely concerned with β-carotene. Among 50 different carotenoids β-carotene is most powerful singlet oxygen quencher and also important because of its conversion to retinol which is also an antioxidant. It is found to have relation in different diseases like in risk of disease development and consequences of diseases. A rapid and simple method was established in which no cold environment was needed, but performed in the dark as all viols used were wrapped with aluminium foil (Kandar et al., 2013). The method was isocratic with flow rate 1mL/min while wavelength was 455 nm. Methanol: Acetonitrile: Chloroform: Tetrahydrofuran were used in a ratio of 50: 20: 25: 5 for mobile phase. Run time was as short as 7.5 minutes with clear peak. Coefficient of variance is presented in table for interday and intraday reproducibility. The method was applied to determine β-carotene status in local healthy population. A total of 100 male and female individuals of the Pakistani population show β-carotene concentration to be 0.61 ± 0.07 µg/mL while concentration of male (0.56 ± 0.04
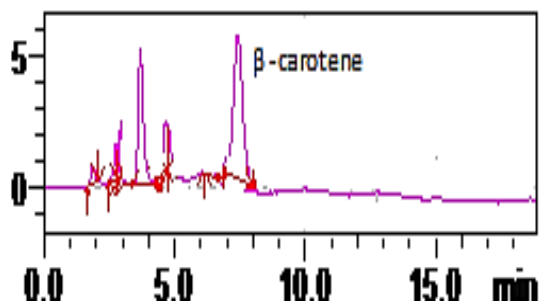
Fig. 1: Representative chromatogram showing the peak for beta carotene, while run time and wave lengths was 7.5 minute 455 nm; respectively

$$y = 13935x + 38566$$
$$R^2 = 0.9835$$
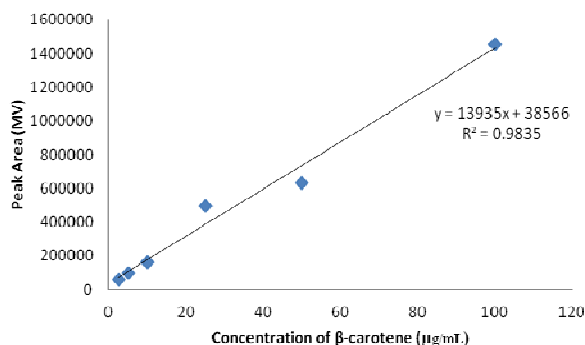
Fig. 2: Standard curve for beta carotene prepared in the mobile phase and further diluted to form standard samples having different concentrations (2.5-100 μg/mL)

Table 1: Interday and Intraday reproducibility

Reproducibility	Concentration (mg/mL)		
	Added	Found (Mean)	Coefficient of variance
Standards	2.5	2.54	6%
	5	5.02	
	10	10.06	7%
	25	25.07	
	50	50.04	5%
	100	100.1	
Intraday	5	5.02	6%
reproducibility	25	25.07	4%
Quality control	50	50.25	7%
Interday	5	5.09	
reproducibility	25	25.04	
Quality control	50	50.11	

μg/mL) was less than female (0.66 ± 0.06 μg/mL) may depend upon dietary intake of carotene rich sources for example carrots. Some epidemiologic studies illustrate mixed findings for β-carotene levels (Ching et al., 2002). A French group has verified that low serum beta-carotene level is found to have greater risk in various forms of cancer. Leukemia and lymphoma, were linked with serum beta-carotene level in epidemiological studies (Mukherjee et al., 2011).

Vitamin A is synthesized by cleavage of beta-carotene. Activity of Glutathione S Transferase was found to be low with vitamin A deficiency and it increases progressively with vitamin A availability (Harrison, 2000). Function of β-carotene, as a potent antioxidant and possible cancer protective agent is suggested by many workers. It reduces cancer risk by preventing reactive oxygen species tissue damage. Studies conducted in China and France have illustrated that lower concentration of serum beta-carotene is a risk factor in different forms of cancers such as leukemia, lymphoma, bone and renal tumors (Liu et al., 1998; Clerici et al., 2004).

There are well documented studies which indicate that adequate plasma concentrations of vitamin E and β-carotene inhibit oxidation of low density lipoproteins, and thus decrease the risk for the development of atherosclerosis, cardiovascular diseases, cancers and age-related muscular degeneration (Grzelinska et al., 2007).

Conclusion

A simple and rapid method for solely β- carotene determination was established with CV less than 10%. It was simple, because no cold environment was needed and rapid as run time is as short as 7.5 minutes. Blood concentration of β-carotene in female was found higher as compared to male subjects by this method having a specific mobile phase composition (50:20:25:5) Methanol: Acetonitrile: Chloroform: Tetrahydrofuran.

REFERENCES

Ching S, D Ingram, R Hahnel, J Beilby and E Rossi, 2002. Serum levels of micronutrients, antioxidants and total antioxidant status predict risk of breast cancer in a case control study. The Journal of Nutrition, 132: 303-306.

Clerici C, D Castellani, G Russo, S Fiorucci, G Sabatino, V Giulino, G Genbli, O Morelli, P Raffo, M Baldoni, A Morelli and S Toma, 2004. Treatment with all trans retinoic acid plus tamoxifen and vitamin E in advanced hepatocellular carcinoma. Anticancer research, 24: 1255-1260.

Devasagayam TPA, JC Tilak, KK Boloor, KS Sane, SS Ghaskadbi and RD Lele, 2004. Free Radicals and Antioxidants in Human Health: Current Status and Future Prospects. Journal of Association of Physicians in India, 52: 794-804.

Grzelińska Z, J Gromadzińska, R Świercz and W Wąsowicz, 2007. Plasma Concentrations of Vitamin E, Vitamin A and β-Carotene in Healthy Men. Polish Journal of Environmental Studies, 16: 209-213.

Harrison EH, 2000. Lipases and carboxyesterases: possible role in the hepatic utilization of vitamin A. Journal of Nutrition, 130: 340-344.

Hercberg S, P Galan, P Preziosi, M Alfarez and C Vazquez. 1998. The potential role of antioxidant vitamins in preventing cardiovascular diseases and cancers. Nutrition, 14: 513-520.

Hostani K and M Kitagawa. 2003. Improved simultaneous determination method of beta-carotene and retinol with saponification in human serum and rat liver. Journal of Chromatograohy B: Analytical Technologies in Biomedical and Life Science, 791: 305-313.

Kandar R, P Novotana and P Drabkova, 2013. Determination of Retinol, -Tocopherol, Lycopene, and -Carotene in Human Plasma Using HPLC with UV-Vis Detection: Application to a Clinical Study. Journal of Chemistry, 2013: 460242.

Lee BL and CN Ong 2009. Comprehensive high-performance liquid chromatographic method for the measurements of lipophilic antioxidants in human plasma. Journal of Chromatography A. 1216: 3131-3137.

Liu G, M Wu, G Levi and N Ferrari, 1998. Inhibition of cancer cell growth by all trans retinoic acid and its analogue N-(4-hydroxyphenyl) retinamide: a possible mechanism of action via regulation of retinoid receptors expression. International Journal of Cancer, 78: 248-254.

Mittal RD and B Mittal, 2004. Correlation of serum retinol and its relation with lipid profile in Indian cancer patients. Indian Journal of Clinical Biochemistry, 19: 36-39.

Mukherjee B, MK Ghosh, CM Hossain, NJ Pharm and H Manage, 2011. Anticancer potential of vitamin A and beta-carotene: Mechanistic Approach, 2: 1-12.

Poppel GV and RA Goldbohm, 1995. Epidemiologic evidence for β-carotene and cancer prevention. American Journal of Clinical Nutrition, 62: 1393- 1402.

Rajendran V, YS Pu and BH Chen, 2005. An improved HPLC method for determination of carotenoids in human serum. Journal of Chromatograohy B: Analytical Technologies in Biomedical and Life Science, 824: 99-106.

Thibeault D, H Su, E MacNamara and H M Schipper. 2009. Isocratic rapid liquid chromatographic method for simultaneous determination of carotenoids, retinol, and tocopherols in human serum. Journal of Chromatograohy B: Analytical Technologies in Biomedical and Life Science, 877: 10770-1083.

Zhao B, SY Tham, J Lu, MH Lai, LKH Lee, SN Moochhala, 2004. Simulataneous determination of vitamin C, E and β-carotene in human plasma by high performance liquid chromatography with photodiode array detection. Journal of Pharmacy and Pharmaceutical Sciences, 7: 200-204.