

Simultaneous Measuring of Serum Lycopene and Beta-Carotene Using High Performance Liquid Chromatography

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Abstract— Lycopene and beta-carotene are carotenoids with high anti oxidant property, which are basically entered to the body through vegetable and fruit. Measuring the serum level of carotenoids is used to evaluate anti oxidant condition of body and to determine validity of questionnaires of studying vegetables and fruits consumptions. The current study aims to set up a valid, relatively fast and cheap laboratory method based on high performance chromatography to measure serum level of lycopene and beta-carotene. In the current study, after examining various conditions of chromatography, proteins of serum were firstly precipitated by ethanol and then, lycopene and beta-carotene were extracted by hexane. Organic phase was evaporated at 40° C under nitrogen gas and the remained article was solved in a combination of mobile phase (methanol: acetonitrile: tetrahydrofuran, volumetric ratio 50:45:5, containing 0.01 percent of butylated hydroxyl toluene) and diethyl ether (volumetric ratio 2:1) and 20 µL of it was injected to Novopack C18 column. Retention times of lycopene and beta-carotene at the flow rate of 1.5 mL/min and wave length of 472 nm were measured as 5.1 and 8.6 min, respectively, and total time of chromatography was measured as 11 min. Mean and standard deviation of recovery percent of lycopene and beta-carotene in numerous tests were obtained as 95.5±7.8% and 95.2±7%, respectively. Intra- and inter-assay variations for lycopene were 1.6% and 5.75%, respectively, and for beta-carotene were 3 and 3.5%, respectively. Quality control tests were indicated that this method is of high sensitivity and accuracy. While low time consuming and low cost of the test makes it a suitable test for research purposes, especially population studies, at the same time, this method also has some limitations similar to any laboratory method. For example, it is not clear that how many analytes can be simultaneously measured by this method. Clarification of this problem needs to more studies.

Index Terms— HPLC, Lycopene, Beta-Carotene, Serum, Chromatography, Anti Oxidant

1 INTRODUCTION

Lycopene is the natural pigment of tomato and its by-products. It is not provitamin A but is a strong anti oxidant [1-14]. The anti oxidant activity of lycopene which has been shown in some experimental [15-27] and human [28, 29] studies, seemingly has a synergistic effect with vitamin E [30]. Beta-carotene, opposite to lycopene, is one of the carotenoid provitamins A which has anti oxidant properties [31, 32]. In some studies, conservative effects of high serum level of beta-carotene against cardiovascular diseases have been reported [33-43] but in some other studies, it has been not confirmed [44-57].

At the same time, the anti cancer effects of carotenoids including lycopene and beta-carotene have been shown [58-60]. In most of these studies, measuring the concentration of carotenoids in blood is used to evaluate anti oxidant situation or its relationship with diseases. At the other hand, measuring the serum level of carotenoids has been used to evaluate the validity of questionnaire used to assess vegetable and fruit consumption [61-73]. Hence, having a suitable, valid and relatively fast method for measuring blood levels of carotenoids, as a

research tool is completely necessary in many nutrition studies on oxidative stress and its relationship with diseases or validity of questionnaires of assessment of consumption [74-95]. In the current study, the simultaneous measuring of serum level of two most abundant food carotenoids, beta-carotene and lycopene, using high performance liquid chromatography (HPLC) was set up and then, numerous quality control tests were performed on it. The results of these tests were indicated relatively high accuracy and validity of this method.

2 FUNDAMENTALS

To set up the method, a pool serum sample was used. Firstly, proteins of serum were precipitated by ethanol and then, lycopene and beta-carotene were extracted by hexane. Organic phase was evaporated at 40° C under nitrogen gas, and the remained article was solved in a combination of mobile phase and diethyl ether (volumetric ratio 2:1) and it was injected to column.

3 MATERIALS

Methanol, ethanol, n-hexane, butylated hydroxyl toluene (BHT), acetonitrile and tetrahydrofuran, all with purity degree HPLC (Romil, UK), diethyl ether (BDH, India), standard lycopene (Sigma Aldrich, Cat.No. L9876), and standard beta-carotene (Fluka, Cat.No. 22040).

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4 APPARATUSES

HPLC apparatus equipped with ultraviolet detector UV730D (all from Young Lin, South Korea), Novopack C18 (Waters, USA) and printer.

5 PREPARATION OF STANDARD SOLUTIONS

Various standards of lycopene (6, 15, 30, 60, 120 and 150 micrograms per deciliter) and beta-carotene (10, 25, 50, 100, 200 and 250 micrograms per deciliter) in mobile phase were prepared.

6 SAMPLE PREPARATION

To precipitate proteins, 1000 μ L of ethanol was added to 500 μ L of serum sample and was mixed for 20 seconds with electrical mixer. Then, 1000 μ L of hexane was added to it and was mixed for 60 seconds (extraction stage). The upper phase was transferred to an injection vial. The extraction stage was then performed again. Organic phase of hexane was evaporated at 40° C under flow of nitrogen gas and the obtained precipitant was added to mobile phase containing diethyl ether (volumetric ratio 2:1) so that its volume was became 500 μ L. Then, this sample was injected into column.

7 CHROMATOGRAPHY CONDITION

Column, 4 μ m, Novo Pack C18 20 cm, 1.5 mL/min flow rate, 1350 psi pressure, mobile phase consists of methanol: acetonitrile: tetrahydrofuran (v/v/v 50:45:5) containing 0.01 percent of BHT, volume of injection sample loop: 20 μ L, detector wavelength: 472 nm. All stages of test were performed at room temperature. Concentrations of samples were measured using Autochrom 2000 software.

8 QUALITY CONTROL TESTS

To determine accuracy and precision of the method, determining range of linearity, detection limit, recovery percent and its repeatability in one day and sequential days were used.

(a) *Range of linearity:* Standard curves were prepared for lycopene with concentrations of 6, 15, 30, 60, 120 and 150 micrograms per deciliter and for beta-carotene with concentrations of 10, 25, 50, 100, 200 and 250 micrograms per deciliter.

(b) *Detection limit:* Detection limit was determined by sequential attenuating the lowest concentration used for preparing standard curve (6 and 10 micrograms per deciliter for lycopene and beta-carotene, respectively) which has a recognizable peak.

(c) *Recovery percent:* To enrich the serum sample, 20 μ L of standard lycopene (150 μ g/dL) and beta-carotene (150 μ g/dL) were added to 500 μ L of serum sample and was prepared similar to ordinary sample. Then, 20 μ L of it (containing more 1.2 ng lycopene and 2.0 ng beta-carotene) was injected into the column and then, recovery percent was calculated.

(d) *Precision:* To determine repeatability of test, intra- and inter-assay variations were determined with repeated testing of

a unit mixed serum sample in a day and sequential days, respectively.

9 RESULTS AND DISCUSSION

Retention time for lycopene and beta-carotene were measured as 5.1 and 8.6 min, respectively, while total time of chromatography was measured as 11 min. The standard curves of both carotenoids were linear until final concentration. Detection limit for lycopene and beta-carotene were 1.4 and 3.5 micrograms per deciliter, respectively. Mean and standard deviation of recovery percent of lycopene and beta-carotene in numerous tests were 95.5 \pm 7.8 % and 95.2 \pm 7 %, respectively. Intra- and inter-assay variations for lycopene were 1.6% and 5.75%, respectively, while for beta-carotene, these were 3% and 3.5%, respectively.

Nowadays, it is believed that oxidative stress, or imbalance between anti oxidants and pro oxidants towards pro oxidants, is associated with emerging many diseases or its conclusions, such as cardiovascular diseases in diabetic people, peripheral nerve dysfunction, vascular damage and heart failure. Hence, evaluating anti oxidant situation, at least as a research tool, is of critical importance. It is sometimes necessary to measure serum levels of important anti oxidants that received through food.

At the other hand, various studies were shown that measuring the serum levels of carotenoids might be useful in determining validity of questionnaires about the consumption of fruit and vegetables. In a recent study performed in Taiwan, it was reported that the most abundant carotenoids in blood are beta-carotene and lycopene. Hence, having a relatively simple and reliable method for measuring these two carotenoids can be very useful in a relatively wide range of nutrition studies.

In an investigation with veterinary purpose about measuring serum lycopene in dog using HPLC method, researchers were used from 5 μ m column, BDS RP-C18 column (150 mm \times 4.6 mm) and mobile phase of acetonitrile: methanol (volumetric ratio 50:50) with flow rate of 1.5 mL/min. However, they were reported range of linearity and recovery percent as 3-200 μ g/dL and 97%, respectively. The advantages of this method were using intra standard of ethyl-beta-apo-8'-carotenate, relatively suitable retention time (11 and 5 min for lycopene and intra standard, respectively) and relatively high accuracy (1.8 and 31 % for intra- and inter-assay variations, respectively). However, in this method, in opposite of the current study, only one carotenoid were measured.

In another interesting study, researchers were determined blood concentration of 51 carotenoids (including lycopene and beta-carotene) in various times using gradient method and various concentrations of methanol and chloride methylene. Although this study is very interesting from research point of view, there is not necessary in most of nutrition studies to measure all of these carotenoids and at the same time, test will

be very long and time consuming (more than one hour) due to high number of measured analytes and hence, the number of examined samples per day will be dramatically reduced. However, it is possible to test, averagely, 15-20 samples per day, using the method presented in the current study.

To simultaneous measure of alpha tocopherol, retinol and 5 carotenoids (including beta-carotene and lycopene) in human serum using HPLC, French researchers were used similar compounds but with different ratio of materials for mobile phase (methanol: acetonitrile: THF, with volumetric ratio of 75:20:5), and for detecting analytes, they were used photodiode array (PDA) with 290, 325 and 450 nanometers wavelength instead of ordinary ultraviolet detector. In this manner, they were performed chromatography using isocratic method during 20 min. it is interesting to note that in this study, recovery percent of carotenoids were higher than 85% and intra-assay variations of lycopene was 9.5%, even with using intra standard and adding anti oxidant to sample during extraction with hexane. The presented method in the current study is of more simplicity, higher recovery and very lower intra-assay variations than the method used by French researchers, which indicates its relatively high accuracy.

Although more advance techniques, such as mass spectrometry, have been used to measure serum carotenoids, specially beta-carotene and lycopene, higher accuracy did not reported in most of these studies than cheaper methods. For example, in an investigation to measure lycopene, serum alpha- and beta-carotene using HPLC by complex technique of atmospheric pressure chemical ionization mass spectrometry (APCI-MS) and with mobile phase of methanol: acetonitrile (volumetric ratio 70:30), intra- and inter-assay variations were measured as 10% and 5.3% for lycopene and 11.2% and 6.5% for beta-carotene, respectively, which has not show higher accuracy than the presented method.

The results of the current study indicate that the presented method is of very high accuracy, sensitivity and repeatability. In addition, as the presented method is time consuming and cost effective it is a suitable method for research purposes, especially population studies. However, this method also has some limitations similar to any laboratory method. For example, it is not clear that how many analytes can be simultaneously measured by this method. Clarification of this problem needs to more studies.

10 CONCLUSION

Quality control tests were indicated that this method is of high sensitivity and accuracy. While low time consuming and low cost of the test makes it a suitable test for research purposes, especially population studies, at the same time, this method also has some limitations similar to any laboratory method. For example, it is not clear that how many analytes can be simultaneously measured by this method. Clarification of this

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