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Determination of Major Carotenoids in Human Serum by Liquid Chromatography

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ABSTRACT

A simple and rapid high performance liquid chromatographic (HPLC) method was developed to determine the major carotenoids in human serum. Lutein, β-carotene and lycopene were separated within 20 min using a C18 column and a mobile phase of methanol/methylene chloride (95:5, v/v) with sample solvent methanol/methylene chloride (45:55, v/v), detection at 476 nm and flow rate at 0.8 mL/min. A high recovery was achieved with this method, which amounted to 92, 90 and 87% for lutein, β-carotene and lycopene, respectively, while the detection limit was 5.0, 10.0 and 4.0 ng/mL. This method was applied to determine the major carotenoids in human serum samples collected from an elderly care center. A total of 62 samples were measured, and in most cases, β-carotene (17.5-1105.7 ng/mL) was present in the highest amount, followed by lutein (10.6-182.3 ng/mL) and lycopene (0-402 ng/mL).

Key words: carotenoids, human serum, HPLC

INTRODUCTION

In the past decade the role of carotenoids in the prevention of chronic diseases such as bladder cancer and prostate cancer has received considerable attention[1,2,3]. Because of the presence of long-chain conjugated carbon-carbon double bonds, carotenoids have been reported to exhibit antioxidative activity[4]. For instance, β-carotene may minimize the attack of free radicals to cells during lipid oxidation through the conversion of a highly reactive singlet oxygen to a more stable triplet oxygen[5]. Although both β-carotene and lycopene possess the same number of conjugated double bonds, the antioxidative activity of the former was shown to be inferior to the latter[6]. Apparently this could be attributed to the greater resonance effect of lycopene due to the coplanarity of 11 conjugated double bonds.

Numerous studies have indicated that lycopene may reduce the risk of bladder cancer[1], prostate cancer[3], gastric cancer[7] and breast cancer[8]. Interestingly, the serum in HIV-infected patients was found to have a lower level of lycopene[9]. Thus, it is possible to diminish the risk of chronic diseases by elevating the level of lycopene in human serum. In addition, the application of lutein in the treatment of age-related macular degeneration is well documented[10].

In view of the impact of these carotenoids to human health, a precise method for their simultaneous determination in human serum is important. The concentrations of lycopene, β-carotene and lutein in human plasma were found to be about 0.16 ± 0.07, 0.74 ± 0.44 and 0.37 ± 0.14 μmol/L[11], respectively. However, no information is available as to the contents of major carotenoids in human plasma in Taiwan. Most published methods employed a ternary or binary gradient solvent system to separate carotenoids in serum. In this study we tried to use an isocratic binary solvent system to separate the major carotenoids in the serum. The objective of this study was to develop a simple and rapid method for the determination of lutein, β-carotene and lycopene in human serum samples collected from an elderly care center by HPLC.

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MATERIALS AND METHODS

I. Materials

A C18 column (Hypersil 5-19626 : 150 × 4.6 mm i.d., 5 μm particle) was purchased from Thermo Electron Co. (Bellefonte, PA, USA) and a C30 column (YMCRP 30: 250 × 4.6 mm i.d., 5 μm particle) was from Waters Corp. (MA, USA). All-trans-lycopene standard was from Extrasynthese Co. (France), all-trans-lutein and all-trans-β-carotene standards were from Sigma Co. (St. Louis, MO, USA). The HPLC-grade solvents, including methanol, acetonitrile, methylene chloride, butanol, and hexane were obtained from Mallinckrodt Co. (Paris, Ky, USA). Deionized water was prepared from a Milli-Q water purification system (Millipore Co., Bedford, MA, USA).

II. *Instrumentation*

The HPLC system is composed of a Phenomenex DG-440 degasser (Phenomenex Co., Torrance, CA, USA), a Rheodyne model 7161 injector (Rheodyne Co., CA, USA), an Agilent model 1100 pump (Agilent Co., CA, USA), an Agilent model 1100 UV-VIS detector and a Jasco MD-915 photodiode-array detector (Tokyo, Japan). A Chem-station

software was used to process data. The N-1 rotary evaporator was from Eyela Co. (Tokyo, Japan). The 2210R-DTH supersonic shaker was from Branson Co. (Danbury, CA, USA). The Sorvall RCSC high-speed centrifuge was from Du Pont Co. (Wilmington, DL, USA).

III. *Extraction of Carotenoids in Human Serum*

The blood samples were collected from an elderly care center located in the suburb of Taipei. A total of 62 samples were collected, transported to the laboratory immediately on the same day, and serum was separated and stored in a freezer (-70°C) until use. A method similar to that described by Talwar *et al*.[12] was used for extraction of carotenoids from serum. Two-hundred μL serum was placed in a centrifuge tube. One mL of distilled water and 70 μL of ethanol solution containing 0.01% ascorbic acid were added for the precipitation of protein and protection of carotenoids. Two mL hexane was added for the extraction of carotenoids, and the mixture was centrifuged at 2500 rpm for 20 min, after which the hexane layer was collected and evaporated to dryness under nitrogen. The residue was dissolved in 100 μL of methylene chloride/methanol (45:55, v/v) and filtered through a 0.2 μm membrane filter. Twenty μL was injected into HPLC.

IV. *HPLC Analysis of Carotenoids in Human Serum*

Various binary and ternary solvent systems were compared with respect to the separation efficiency of lutein, β-carotene and lycopene. Two binary solvent systems in different proportions, including methanol/methylene chloride (99:1, 97:3 and 95:5, v/v) and water/acetonitrile (5:95, 10:90 and 20:80, v/v) were used. Likewise, two ternary solvent systems in different proportions, including n-butanol/acetonitrile/methylene chloride (30:70:10, v/v/v) and iso-butanol/acetonitrile/methylene chloride (25:70:5, 25:70:10 and 25:70:20, v/v/v) were used. The solvent strength of each mobile phase was carefully controlled by calculating the polarity index. Two analytical columns C18 and C30 were used for comparison. The flow rate was 0.8 mL/min and column temperature was 25°C with detection at 476 nm and sensitivity at 0.005 AUFS. The major carotenoids, lutein, β-carotene and lycopene in serum samples were identified by comparing the retention times and absorption spectra of unknown peaks with reference standards and cochromatography with added standards. Because of the absence of a suitable internal standard, the major carotenoids were quantified using absolute calibration curves. Five concentrations ranging 0-1.0 μg/mL were prepared for lutein, β-carotene and lycopene. After injection into HPLC, the calibration curve of each carotenoid was made by plotting peak area against concentration. High correlation coefficients (R^2) were found for lutein, β-carotene and lycopene, which reached to 0.9998, 0.9992 and 0.9990, respectively (Figure 1). The amounts of carotenoids were calculated from the following regression equations: y = 54.058x-0.2314 for lutein, y = 108.34 x + 0.372 for β-carotene and y = 152.83 x - 1.1421 for lycopene, where y denotes peak area and x denotes concentration. Duplicate analyses were carried out and the data were expressed as means ± standard deviations.

V. *Recovery Study*

Human serum was spiked with 20 μL of combined carotenoid standards with a concentration of 20 ng/mL each. The spiked serum was then extracted as described above. After HPLC analysis, the recovery of each major carotenoid was obtained by dividing the calculated concentration by the added concentration. Triplicate analyses were performed and the mean value was determined.

RESULTS AND DISCUSSION

I. *Extraction of Carotenoids in Human Serum*

The extraction method used in this study is simple and

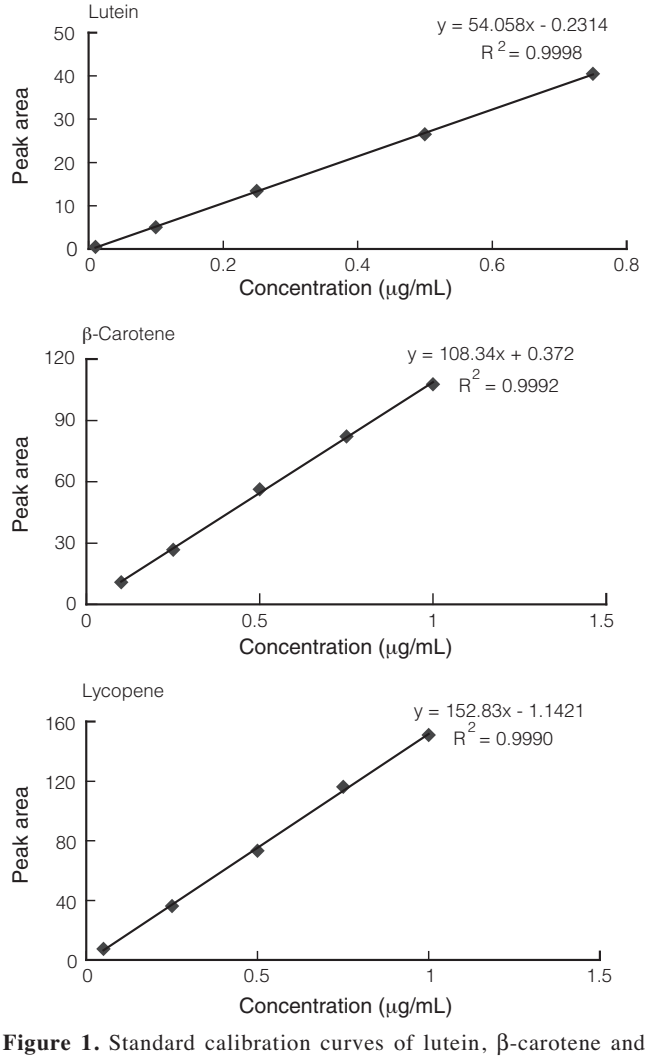

Figure 1. Standard calibration curves of lutein, β-carotene and lycopene.

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rapid, which requires 20 min for complete extraction of carotenoids. Ascorbic acid was added to the extraction solvent because it has been reported that it could minimize isomerization and oxidation loss of carotenoids during extraction and subsequent evaporation[12]. A sample solvent of methylene chloride/methanol (45:55, v/v) was used to dissolve the residue. According to a study by Chen and Chen[13], an appropriate sample solvent has to be selected carefully because it may change the polarity of mobile phase and thus affect the separation efficiency of carotenoids. The extraction efficiency was accomplished by a high recovery, which reached to 92, 90 and 87 % for lutein, β-carotene and lycopene, respectively. These results were similar to a report by Talwar *et al.*[12], who also used ethanol and hexane for extraction of carotenoids and the recoveries for lutein, β-carotene and lycopene were 94, 87 and 89%, respectively. Apparently the addition of ascorbic acid to the extraction solvent could enhance the extraction efficiency greatly.

II. *Separation of Carotenoids in Human Serum*

Initially 2 types of columns and various binary and ternary solvent systems were compared with respect to the separation efficiency of lutein, β-carotene and lycopene in human serum. With an appropriate mobile phase, both C18 and C30 columns could provide good separation of these carotenoids. However, the latter resulted in a much longer retention time (>50 min) than the former. This could be attributed to a greater hydrophobic interaction between C30 stationary phase and the major carotenoids, as reported by Tai and Chen[14]. Thus, a C18 column was selected instead of a C30 column, and the retention time could be drastically reduced from 50 to 20 min. The most appropriate mobile phase for a C18 column was found to be methanol/methylene chloride (95:5,v/v) with sample solvent methanol/methylene chloride (45:55, v/v). This binary solvent system was able to separate lutein, β-carotene and lycopene standards within 20 min (Figure 2). Several minor peaks were also shown on the HPLC chromatogram, which may be due to the presence of carotenoid isomers. Figure 3 shows the HPLC chromatogram of major carotenoids in human serum. Lutein, β-carotene and lycopene were identified based on the criteria described in the method section. Likewise, several unknown peaks were present, which were not identified because of the absence of commercial standards of carotenoid isomers. An adequate resolution was observed for lutein, lycopene and β-carotene, and the retention times were 3.1, 17.5 and 19.9 min, respectively. Talwar *et al.*[12] developed a mobile phase of methanol/acetonitrile/tetrahydrofuran (75:20:5, v/v/v) containing 0.01% ascorbic acid to separate lutein, β-carotene and lycopene within 16 min. However, this solvent system failed to resolve the major carotenoids in human serum in our study, probably because of differences in column length and internal diameter, as well as flow rate and the variety of sample. Also, the ternary solvent system employed is more complicated than the binary solvent system used in our study. In addition, the authors[12] used 450 nm to detect the major carotenoids, which may underestimate the level of lycopene because of a lower response when compared to lutein or β-carotene. As lycopene has been proven to be a more important biological compound than lutein or β-carotene, the amount of lycopene has to be accurately quantified. Thus, in this study the detection wavelength was changed to 476 nm for the simultaneous determination of lycopene, lutein and β-carotene. With the methods of Milne and Botnen[15] and Thurnham *et al.*[16], no lutein was separated and lycopene was difficult to quantify because of its instability to form cis isomers. Several authors also applied gradient solvent systems to separate the major carotenoids in plasma. However, these methods are either lengthy or complicated when compared to isocratic solvent system[17,18].

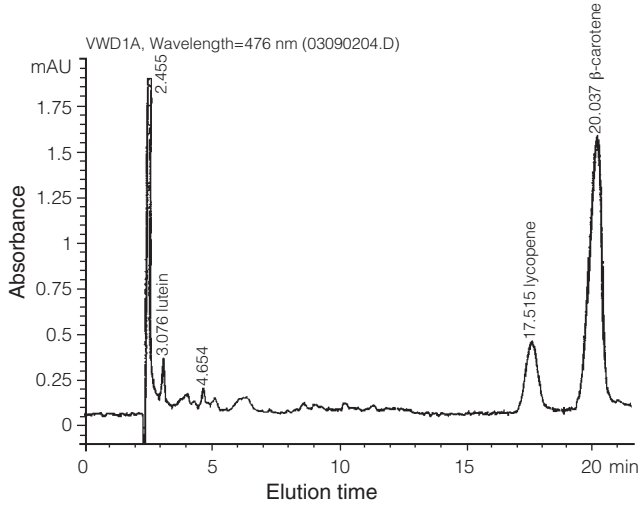

Figure 2. HPLC chromatogram of carotenoid standards. Chromatographic conditions were described in text. Peaks: 1. lutein; 2. lycopene; 3. β-carotene.

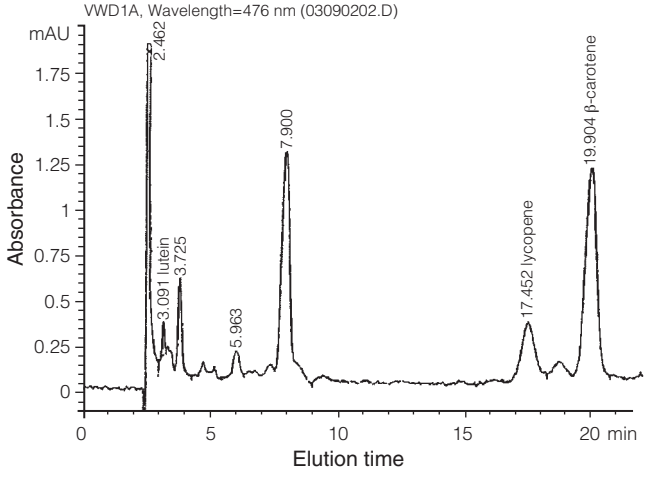

Figure 3. HPLC chromatogram of carotenoids in a human serum sample. Chromatographic conditions were described in text. Peaks: 1. lutein; 2. lycopene; 3. β-carotene.

III. *Quality Control*

The detection limits for lutein, β-carotene and lycopene were 5.0, 10.0 and 4.0 ng/mL, respectively, based on a ratio of signal to noise which is higher than 3. Lycopene was shown to exhibit the lowest detection limit mainly because a higher response was obtained with detection at 476 nm when compared to lutein and β-carotene. For repeatability and reproducibility test, 1 ppm each of lutein, β-carotene and lycopene were injected into HPLC 3 times on the same day and 6 times a week for a total of 4 weeks. The coefficients of variation (CV %) of the intra-day variabilities for lutein, β-carotene and lycopene were 1.87, 1.54 and 2.36%, respectively, while the inter-day variabilities were 3.21, 3.56 and 4.78 %.

IV. *Concentration of Carotenoids in Human Serum*

Table 1 shows the quantitative data of major carotenoids in human serum samples. In most samples, β-carotene showed higher concentrations, whereas both lutein and lycopene showed lower concentrations. Also, a greater range of β-carotene level was found. For example, the highest β-carotene concentration was 1105.7 ng/mL (2.06 μmol/L) while the lowest was 17.5 ng/mL (0.03 μmol/L). For lutein and lycopene, the levels ranged from 10.6-182.3 ng/mL (0.02-0.32 μmol/L) and 0-402 ng/mL (0-0.75 μmol/L), respectively. A large variation existed probably because of difference in diet pattern and absorption of the individual carotenoid. Nevertheless, the mean concentrations of the major carotenoids analyzed in this study were close to those reported by Watzl *et al.*[11], who found that the levels of β-carotene, lutein and lycopene in the human plasma were 0.74, 0.37 and 0.16 μmol/L, respectively. As lycopene was reported to possess a higher antioxidative activity than lutein or β-carotene, the diet pattern for the elderly has to be modified so that the lycopene level in the serum can be elevated for those containing low contents of lycopene. It has been well established that lycopene is the dominate carotenoid in human serum[19,20]. However, in this study this phenomenon was not observed, probably because the tomatoes consumed in the diet by the elderly were not adequately processed. Several reports have demonstrated that the consumption of adequately processed tomatoes such as tomato juice could enhance lycopene bioavailability because more lycopene could be released from the cell matrix of tomatoes[21]. Dietary supplementation with tomato juice was found to increase plasma lycopene significantly, whereas it remained unchanged for those consuming raw tomatoes[20]. Although lycopene possesses no provitamin A activity, it shows other vital biological properties and is superior to β-carotene in terms of suppression of proliferation of cancer cells[22]. Watzl *et al.*[11] conducted a human intervention study to determine the effect of consumption of carotenoid-rich vegetables on the immune system and found that a low-carotenoid diet reduces T-lymphocyte functions, and addition of tomato

Table 1. Concentrations of carotenoids in human serum samples

Sample No	Concentration (ng/mL)[a]		
	Lutein	β-Carotene	Lycopene
1	18.1 ± 1.2	209.2 ± 1.7	52.1 ± 1.0
2	31.8 ± 1.0	291.0 ± 1.0	62.0 ± 0.5
3	15.2 ± 0.3	1105.7 ± 8.2	25.8 ± 0.7
4	21.8 ± 0.6	47.6 ± 0.9	12.0 ± 0.5
5	12.9 ± 0.2	54.7 ± 3.2	13.5 ± 0.4
6	19.0 ± 0.3	50.1 ± 3.1	22.8 ± 1.0
7	35.1 ± 2.3	64.3 ± 2.3	10.2 ± 0.5
8	17.1 ± 2.0	68.8 ± 4.1	20.9 ± 1.7
9	21.7 ± 2.0	77.7 ± 0.6	19.5 ± 0.8
10	37.3 ± 1.4	88.4 ± 0.2	21.6 ± 0.3
11	29.8 ± 1.4	144.5 ± 4.6	15.7 ± 0.6
12	30.8 ± 0.5	128.5 ± 1.3	10.4 ± 0.2
13	41.7 ± 0.3	155.4 ± 3.0	22.6 ± 0.8
14	182.3 ± 3.6	207.2 ± 3.9	26.9 ± 0.7
15	31.9 ± 1.6	136.5 ± 1.2	44.5 ± 0.9
16	33.3 ± 0.2	123.6 ± 3.1	193.4 ± 0.5
17	10.9 ± 0.5	18.0 ± 0.2	13.1 ± 0.1
18	10.6 ± 0.2	30.4 ± 1.3	8.0 ± 0.2
19	39.3 ± 0.7	83.1 ± 3.0	16.0 ± 0.6
20	32.6 ± 0.5	78.3 ± 0.7	8.1 ± 0.2
21	18.9 ± 1.0	67.7 ± 1.0	12.4 ± 0.5
22	15.3 ± 0.3	98.8 ± 0.7	43.7 ± 0.6
23	32.4 ± 0.3	182.1 ± 2.3	62.6 ± 1.2
24	22.0 ± 1.0	190.6 ± 3.6	22.4 ± 0.6
25	50.9 ± 2.5	166.5 ± 1.1	402.0 ± 1.1
26	31.7 ± 0.3	145.3 ± 0.3	21.6 ± 0.3
27	16.2 ± 0.3	41.5 ± 0.5	22.7 ± 0.2
28	24.1 ± 0.3	130.0 ± 0.7	13.9 ± 0.2
29	31.0 ± 0.7	188.6 ± 0.5	78.1 ± 0.7
30	29.1 ± 0.7	63.8 ± 1.2	ND[b]
31	23.6 ± 0.3	40.1 ± 0.2	5.7 ± 0.4
32	54.2 ± 4.9	341.8 ± 0.9	57.7 ± 0.9
33	32.6 ± 2.3	69.7 ± 0.9	24.6 ± 0.5
34	62.1 ± 3.5	113.1 ± 9.4	112.3 ± 1.6
35	13.8 ± 2.6	24.8 ± 2.1	11.0 ± 1.0
36	67.1 ± 0.7	228.5 ± 3.7	40.7 ± 2.4
37	25.4 ± 4.4	151.0 ± 2.1	87.2 ± 1.7
38	64.1 ± 1.8	22.5 ± 0.3	14.3 ± 0.2
39	71.5 ± 1.4	213.7 ± 9.2	27.8 ± 2.5
40	15.9 ± 1.4	73.1 ± 1.3	35.0 ± 1.4
41	124.5 ± 2.6	144.2 ± 2.2	46.0 ± 1.8
42	32.6 ± 2.3	89.4 ± 1.4	10.3 ± 0.6
43	24.9 ± 5.0	149.1 ± 1.4	42.6 ± 1.7
44	13.6 ± 0.9	129.6 ± 11.6	44.6 ± 0.5
45	29.4 ± 0.5	63.8 ± 1.7	5.3 ± 0.6
46	22.0 ± 0.5	43.1 ± 0.6	33.4 ± 1.4
47	29.9 ± 1.9	32.4 ± 3.8	81.4 ± 1.8
48	14.1 ± 1.0	29.6 ± 4.5	8.0 ± 0.6
49	17.1 ± 0.2	57.0 ± 0.6	10 ± 0.4
50	11.3 ± 0.5	24.1 ± 0.3	14.9 ± 0.6
51	ND	17.5 ± 0.5	ND
52	44.5 ± 1.2	51.8 ± 0.7	17.3 ± 0.2
53	33.1 ± 4.5	58.5 ± 1.3	103.5 ± 2.2
54	17.4 ± 0.5	28.0 ± 0.5	11.1 ± 0.9
55	22.4 ± 0.9	66.0 ± 1.8	6.5 ± 0.2
56	45.6 ± 1.4	257.4 ± 0.5	98.5 ± 1.5
57	23.6 ± 0.7	140.0 ± 1.0	100.3 ± 1.1
58	11.8 ± 0.5	61.9 ± 1.6	29.1 ± 1.2
59	50.7 ± 1.4	114.3 ± 0.9	32.8 ± 1.1
60	22.7 ± 1.2	147.6 ± 1.2	59.4 ± 2.5
61	29.9 ± 1.0	130.3 ± 2.9	58.3 ± 0.8
62	19.4 ± 2.1	114.2 ± 2.2	32.3 ± 1.3

[a]Average of duplicate analyses ± standard deviation.
[b]ND: not detected.

juice restores these functions. In another study, Watzl *et al*.[23] further demonstrated that after consumption of tomato juice for 8 weeks, the concentrations of lycopene and β-carotene increased by 0.36 and 0.31 μmol/L, respectively. In contrast, the level of lutein decreased by 0.07 μmol/L. This result clearly demonstrates that a lycopene-rich diet is crucial to enhance immune response of the elderly. Further research is necessary to study the distribution of various carotenoids in tissues of human body.

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