

## Simultaneous determination of vitamins C, E and $\beta$ -carotene in human plasma by high-performance liquid chromatography with photodiode-array detection.

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**Abstract Purpose:** To develop a high-performance liquid chromatography (HPLC) method with photodiode-array ultraviolet detection for the simultaneous determination of vitamin C, vitamin E and  $\beta$ -carotene. **Methods:** Following liquid-phase extraction from the human plasma samples, these three vitamins were successfully separated on the LiChrospher 100 RP-18 column (125  $\times$  4 mm I.D.; particle size, 5  $\mu$ m) at a flow-rate of 1.2 ml/min, with a mobile phase of methanol-acetonitrile-tetrahydrofuran (75: 20: 5, v/v/v). **Results:** The limits of quantitation were 100, 0.25 and 0.25  $\mu$ g/ml for vitamin C, vitamin E and  $\beta$ -carotene respectively. The method is linear over the studied range of 0.25 to 5  $\mu$ g/ml for vitamin E and  $\beta$ -carotene and 100 to 5000  $\mu$ g/ml for vitamin C. The extraction recoveries were greater than 83% for these three vitamins. The within day and between-day precision of the analysis did not exceed 15.3 and 16.2%, respectively. **Conclusion:** A suitable method to determine the concentration of vitamin C, vitamin E and  $\beta$ -carotene following oral administration of antioxidant supplement capsules to a healthy Chinese volunteer.

### INTRODUCTION

Free radicals are highly reactive molecules that react with and damage cells throughout the body. They are suspected of causing cardiovascular disease, cancer, neurological disorders, cataracts, arthritis, aging and other conditions such as muscle damage and fatigue that could inhibit performance (1, 2). Antioxidants are molecules, which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. There are several enzyme systems within the body that scavenge free radicals, whereby the principal micronutrient (vitamin) antioxidants are vitamin C, vitamin E and  $\beta$ -carotene. The body cannot manufacture

these micronutrients so they must be supplied in the diet (2).

Many researchers believe that vitamin supplements can dramatically reduce free radical damage, prevent and delay the onset of chronic degenerative diseases, and possibly extend lifespan (3). Numerous epidemiological studies have demonstrated an association between higher intakes or higher blood concentrations of certain antioxidants and a lower incidence of certain degenerative diseases (4, 5). Clinical studies have shown that supplemental levels of antioxidant vitamins reduce an individual's risk for certain cancers and cardiovascular diseases (6, 7). Moreover, studies have shown that fruit and vegetable consumption (the major source of antioxidant nutrients) has a protective effect against cancer (8). Evidence also suggests that vitamins C, E and  $\beta$ -carotene supplementation have ergogenic or performance enhancing effects (9).

High-performance liquid chromatography (HPLC) combined with a UV-Vis detector is the most common method for identification and quantification of antioxidant vitamins in biological fluids. Several HPLC methods have been presented for the determination of vitamin C in serum or plasma (10, 11, 12, 18). The procedures for simultaneous measurement of vitamin E and  $\beta$ -carotene by HPLC have also been described (13, 14, 17). Recently, an isocratic liquid chromatographic method was reported for the simultaneous determination of vitamins C, E and  $\beta$ -carotene in pharmaceutical preparations (15). However, no reports for the simultaneous determination of these three antioxidant vitamins in human biological fluids were described. This is attributed to the extraction and reconstitution difficulties from biological fluids that are linked to their chemical properties (Vitamin C is water-soluble; Vitamin E and  $\beta$ -carotene are fat-soluble).

In this paper, we report a simple, sensitive and reliable

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isocratic HPLC assay for the simultaneous determination of antioxidant vitamins (vitamin C, vitamin E and  $\beta$ -carotene) in human plasma using photodiode-array detection. This method has been successfully employed to monitor plasma vitamin C, vitamin E and  $\beta$ -carotene levels following oral administration of antioxidant supplement capsules to a healthy Chinese volunteer.

## METHODS

### *Chemicals and materials:*

Vitamin C (L-ascorbic acid, purity: 99%), vitamin E [( $\pm$ )-alpha-tocopherol, purity: 95%] and  $\beta$ -carotene (purity: 97%) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Methanol, acetonitrile and tetrahydrofuran were obtained from Merck KGaA (Darmstadt, Germany). All solvents were of HPLC grade. All the reagents were used without further purification. Deionized water, purified by Milli Q system (Millipore, Milford, MA, USA), was used throughout the study. Stock solutions of vitamin E and  $\beta$ -carotene were prepared at 10 mg/ml in chloroform. Stock solution of vitamin C was prepared at 10 mg/ml in methanol. All stock solutions were protected from light and stored at  $-20^{\circ}\text{C}$ . The stock solutions were further diluted with methanol to give a series of working standards. The working solutions for spiking blank human plasma samples were prepared fresh daily.

### *Instrumentation:*

The HPLC system (Waters 2690 Separation Module) consisted of a Waters 600E multisolvent delivery system pump, a Waters Ultra WISP 715 autoinjector, and a Waters 996 diode-array detection system set in a range of 200 – 400 nm (all from Waters, Milford, MA, USA). Vitamins C, E and beta-carotene were separated on the LiChrospher 100 RP-18 column ( $125 \times 4$  mm I.D.; particle size, 5  $\mu\text{m}$ ) from Merck KGaA (Darmstadt, Germany), with a mobile phase of methanol-acetonitrile-tetrahydrofuran (75: 20: 5, v/v/v) at a flow-rate of 1.2 ml/min.

### *Sample preparation:*

Human plasma samples were spiked with concentrations ranging from 100 to 5000  $\mu\text{g/ml}$  for vitamin C and from 0.25 to 5  $\mu\text{g/ml}$  for vitamin E and  $\beta$ -carotene. Vitamin C in plasma was extracted as follows: plasma protein was precipitated with 60% methanol and 1mM

EDTA. Plasma (100 $\mu\text{l}$ ) was mixed with 400 $\mu\text{l}$  of 60% methanol/EDTA, incubated for 10min at  $4^{\circ}\text{C}$  before centrifuging at 12,000 rpm for 8min. The clear phase was transferred to another polypropylene tube and evaporated to dryness under nitrogen. The dried extracts were dissolved in 100 $\mu\text{l}$  of methanol.

Vitamin E and  $\beta$ -carotene in plasma were extracted as follows: 100 $\mu\text{l}$  of plasma was deproteinized with 100 $\mu\text{l}$  of ethanol and was extracted with 600 $\mu\text{l}$  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 100 $\mu\text{l}$  of methanol. All reconstituted antioxidants were mixed together before injecting into the HPLC system.

### *Assay validation:*

Samples were quantified using peak area of vitamin C, vitamin E and  $\beta$ -carotene. Standard calibration curves were constructed by spiked drug-free pooled human plasma with a known amount of vitamin C, vitamin E and  $\beta$ -carotene. These plasma standards were also used to determine the extraction recovery, within day and between-day precision ( $n = 5$ ) of the method. The recoveries of vitamin C, vitamin E and  $\beta$ -carotene after liquid-phase extraction were calculated by comparing observed vitamins C, E and  $\beta$ -carotene peak areas in extracted plasma, to those of non-processed standard solutions. Limit of quantitation is based on the lowest concentration validated by the method.

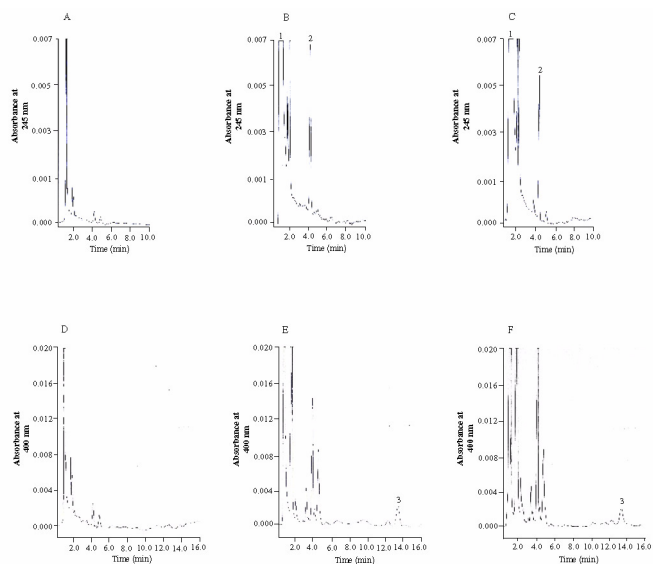
### *Applicability:*

One healthy non-smoking Chinese volunteer (male, aged 25 year) ingested three capsules of antioxidant supplement (Antioxidant Fuel, Twin Laboratories Inc., NY, USA) each day for 40 days. Each capsule contains 333 mg of vitamin C, 266 IU of vitamin E, 8,333 IU of  $\beta$ -carotene, 39 mg of dicalcium phosphate anhydrous, 33  $\mu\text{g}$  of sodium selenate, 10 mg of Co enzyme Q10, 66 mg of N-acetyl-L-cysteine, 33 mg of L-glutathione and 33 mg of alpha-lipoic acid. Venous blood samples were collected just before the first oral dose, and on the 15<sup>th</sup>, 30<sup>th</sup>, 33<sup>rd</sup>, 34<sup>th</sup>, 36<sup>th</sup> and 40<sup>th</sup> day of the study period. The plasma was obtained immediately by centrifugation at 2000 g for 5 min and stored at  $-20^{\circ}\text{C}$  until analysis by HPLC.

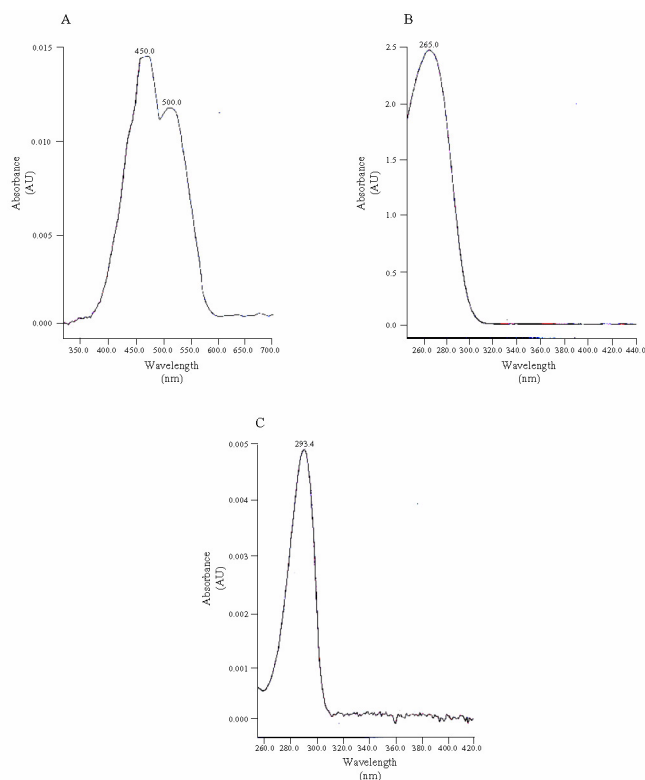
## RESULTS AND DISCUSSION

In the current study, we developed a simple HPLC method for simultaneous determination of antioxidant vitamins (vitamins C, E and  $\beta$ -carotene) in human plasma. The solubility and polarity of the three analytes are vastly different, thus leading to the need for different grades of polar solvents to extract and constitute. After extraction, the final component reconstituents were mixed to enable a single injection into the HPLC. This method utilizes the photodiode-array detector for measuring three vitamins at various wavelengths in a single injection. The photodiode-array detector has an added advantage of effecting a rapid identification of UV-Vis spectra. In addition, peak purity of plasma samples as compared with standards can be monitored by this detection method.

Representative chromatograms of blank and spiked human plasma samples are shown in Figure 1. The wavelength for the detection of vitamins C and E was 245 nm (upper panels); and was switched to 400 nm for  $\beta$ -carotene (lower panels). Figure 2 shows the spectra of the compounds. Under the described conditions, the retention times were 0.92, 4.1 and 13.7 min for vitamin C, vitamin E and  $\beta$ -carotene, respectively. The total run time was 15 min. clean chromatogram shows negligible interference from endogenous substances in plasma sample. Limits of quantitation were 100  $\mu\text{g}/\text{ml}$  for vitamin C, 0.25  $\mu\text{g}/\text{ml}$  for vitamin E and 0.25  $\mu\text{g}/\text{ml}$  for  $\beta$ -carotene. Regression analysis was performed on the calibration curve in plasma. The calibration curves of the analytes obtained for five independent runs were linear within the studied range between 0.25 to 5  $\mu\text{g}/\text{ml}$  for vitamin E and  $\beta$ -carotene, and 100 to 5000  $\mu\text{g}/\text{ml}$  for vitamin C based on the peak area. The regression equations describing the calibration runs were vitamin C,  $y = 500000x + 88306$  ( $R^2 = 0.9918$ ); vitamin E,  $y = 12486x + 7609$  ( $R^2 = 0.9781$ ); and  $\beta$ -carotene,  $y = 45052x + 2844$  ( $R^2 = 0.9929$ ), where  $y$  is the peak area of the antioxidants and  $x$  is the concentration. These studied ranges were found to be appropriate to cover the range of analyte concentration in plasma after oral administration of antioxidant supplement capsules to the healthy Chinese volunteer. The extraction recoveries of standards spiked in plasma samples were calculated. The recoveries of vitamin C, vitamin E and  $\beta$ -carotene within the concentration range of the assay were 83%, 100% and 91% respectively.



**Figure 1:** HPLC chromatograms of blank human plasma (A) and (D), blank human plasma spiked with vitamin C, vitamin E with concentration of 2.5mg/ml and 2.5 $\mu\text{g}/\text{ml}$  respectively (B) and beta-carotene with concentration of 2.5mg/ml (E); and plasma sample from the subject (C) and (F). Peak 1: vitamin C, peak 2: vitamin E, and peak 3: beta-carotene. Chromatogram (A), (B) and (C) were detected at 245nm, whereas (D), (E) and (F) were detected at 400nm.



**Figure 2:** UV absorbance spectra of the standard (A) beta-carotene, (B) vitamin C and (C) vitamin E.

The vitamins under study are unstable and especially sensitive to light, heat, oxygen and peroxide (16). Therefore, special precautions have to be during the experiments. Stock solutions were tested four times from the time of their preparation up to 14 days and the results indicated that the stock solutions were stable within this period. However, the working solutions for spiking blank human plasma samples were freshly prepared from these stock solutions, and used immediately.

The within- and between-day variations ( $n = 5$ ) of the method are given in Table 1. For each level, the repeatability and reproducibility criteria were clearly satisfactory. For the three analytes, within-day variability was lower than 15.3 % while between-day variability did not exceed 16.2 %. Within-day variability averaged 11.0 % for vitamin C, 5.3 % for vitamin E and 5.9 % for  $\beta$ -carotene. Mean between-day variability was 11.6 % for vitamin C, 5.5% for vitamin E and 9.6 % for  $\beta$ -carotene. To minimize analytical variability, analyte concentrations in human plasma were always derived according to the same day standard curve.

**Table 1: Within- and between-day assay variability ( $n = 5$ )**

| Compound          | Added concentration ( $\mu\text{g/ml}$ ) | Measured concentration ( $\mu\text{g/ml}$ ) |        |                  |        |
|-------------------|--|---|--------|------------------|--------|
|                   |  | Within-day                                  |        | Between-day      |        |
|                   |  | Mean $\pm$ SD                               | CV (%) | Mean $\pm$ SD    | CV (%) |
| Vitamin C         | 100                                      | 98 $\pm$ 15                                 | 15.3   | 98 $\pm$ 13      | 13.3   |
|                   | 500                                      | 504 $\pm$ 74                                | 14.7   | 460 $\pm$ 57     | 12.4   |
|                   | 1000                                     | 1100 $\pm$ 158                              | 14.4   | 920 $\pm$ 109    | 11.9   |
|                   | 2500                                     | 2500 $\pm$ 158                              | 6.3    | 2460 $\pm$ 260   | 10.6   |
|                   | 5000                                     | 4680 $\pm$ 190                              | 4.1    | 4920 $\pm$ 470   | 9.6    |
| Vitamin E         | 0.25                                     | 0.248 $\pm$ 0.01                            | 3.2    | 0.255 $\pm$ 0.01 | 2.8    |
|                   | 0.50                                     | 0.53 $\pm$ 0.05                             | 9.4    | 0.49 $\pm$ 0.01  | 1.4    |
|                   | 1.00                                     | 1.08 $\pm$ 0.08                             | 7.1    | 1.10 $\pm$ 0.03  | 2.3    |
|                   | 2.50                                     | 2.56 $\pm$ 0.11                             | 4.5    | 2.50 $\pm$ 0.41  | 16.2   |
|                   | 5.00                                     | 4.72 $\pm$ 0.11                             | 2.3    | 5.10 $\pm$ 0.25  | 4.8    |
| $\beta$ -carotene | 0.25                                     | 0.25 $\pm$ 0.01                             | 5.2    | 0.24 $\pm$ 0.03  | 10.8   |
|                   | 0.50                                     | 0.49 $\pm$ 0.04                             | 7.3    | 0.53 $\pm$ 0.08  | 15.8   |
|                   | 1.00                                     | 0.95 $\pm$ 0.05                             | 5.1    | 0.96 $\pm$ 0.13  | 14.0   |
|                   | 2.50                                     | 2.50 $\pm$ 0.20                             | 8.0    | 2.40 $\pm$ 0.14  | 5.9    |
|                   | 5.00                                     | 4.86 $\pm$ 0.20                             | 4.0    | 5.15 $\pm$ 0.07  | 1.4    |

The applicability of the assay was demonstrated in plasma samples following oral administration of antioxidant supplement capsules to one healthy Chinese volunteer. After liquid-phase extraction, HPLC chromatogram was obtained (Figure. 1). The results of the assay are shown in Table 2. The amount of vitamin C, vitamin E

and  $\beta$ -carotene in plasma was above the limit of detection of the assay.

**Table 2: Blood concentration of antioxidants after ingestion of vitamin supplements at various time points.**

| Compound                                 | Day              |                  |                  |                  |                  |                  | mean $\pm$ SD   |
|--|------------------|------------------|------------------|------------------|------------------|------------------|-----------------|
|  | 15 <sup>th</sup> | 30 <sup>th</sup> | 33 <sup>rd</sup> | 34 <sup>th</sup> | 36 <sup>th</sup> | 40 <sup>th</sup> |                 |
| Vitamin C / (mg/ml)                      | 2.48             | 1.64             | 1.35             | 2.10             | 1.20             | 1.85             | 1.92 $\pm$ 0.59 |
| Vitamin E / ( $\mu\text{g/ml}$ )         | 9.94             | 4.90             | 14.05            | 10.13            | 4.167            | 4.56             | 6.86 $\pm$ 4.68 |
| $\beta$ -carotene / ( $\mu\text{g/ml}$ ) | 2.16             | 1.29             | 2.21             | 1.26             | 0.91             | 0.99             | 1.26 $\pm$ 0.76 |

## CONCLUSIONS

The proposed HPLC method, which combines photodiode-array detection, reaches the optimum performance in terms of selectivity, precision, and accuracy for antioxidant study in humans. The procedure employs a relatively simple liquid phase clean-up procedure for sample preparation. The efficiency is enhanced by the use of only one isocratic chromatographic elution that separates and quantifies the water-soluble vitamin C and fat-soluble vitamin E and  $\beta$ -carotene. This method can be utilized in studies that require monitoring of plasma vitamin C, vitamin E and  $\beta$ -carotene levels after oral administration of antioxidant supplement capsules to human subjects.

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