Original Article

Serum concentrations of micronutrient antioxidants in an adult Arab population

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Serum concentrations of retinol, α-tocopherol, β-carotene and lycopene were measured by reversed-phase high-performance liquid chromatography (r-*P* HPLC) in 260 randomly selected healthy adult Kuwaitis (159 men and 101 women) aged 18–63 years (mean 33.3 years) to established reference ranges of the micronutrient antioxidants. Total cholesterol concentrations were assayed by an enzymatic method to determine α-tocopherol : cholesterol ratios. The mean ± SEM (μmol/L) for retinol, α-tocopherol, β-carotene and lycopene were 1.76 ± 0.02, 20.0 ± 0.5, 0.52 ± 0.03, 0.95 ± 0.05, respectively. Compared to other populations, these data showed, on the whole, ordinary concentrations of β-carotene, comparatively low concentrations of retinol and α-tocopherol and high concentrations of lycopene. Retinol concentrations were similar for both sexes, whereas α-tocopherol concentration was significantly ($P < 0.0001$) lower and the carotenoid levels (β-carotene and lycopene) significantly higher ($P < 0.0001$) in women. Of the micronutrient antioxidants, α-tocopherol was most correlated with cholesterol ($r = 0.492$; $P < 0.0001$). β-Carotene and lycopene were highly correlated with each other ($r = 0.744$, $P < 0.0001$). Age was positively associated with β-carotene ($r = 0.214$, $P = 0.001$) and lycopene ($r = 239$, $P < 0.0001$). Our data enabled us to establish a gender non-specific reference range for retinol and gender-specific reference ranges for α-tocopherol, β-carotene and lycopene.

Key words: Arabs, β-carotene, cholesterol, Kuwait, lycopene, micronutrient antioxidants, retinol, α-tocopherol.

Introduction

Vitamins A (retinol), E (α-tocopherol) and the carotenoids (e.g., β-carotene and lycopene) are micronutrient antioxidants that have an integral role in regulating vital metabolic reactions in the body. Optimal micronutrient antioxidant status is an essential requirement in any population. Evidence compiled from epidemiological studies suggests that α-tocopherol levels and mortality from ischaemic heart disease are inversely related,[1–3] as are serum carotenoid concentrations and the incidence of numerous types of cancer.[4] It has been observed that these micronutrient antioxidants affect immune status.[5,6]

The dietary habits regarding intake of fat, fruits and vegetables may be the dictating factors in the micronutrient antioxidant status of different populations. Consequently, micronutrient antioxidant status may vary among populations.[1]

Impaired micronutrient antioxidant status has been observed in disorders including cholestasis,[7] pancreatic insufficiency[8] and protein calorie malnutrition.[9] Intervention studies have been carried out[10–12] for correction of these deficiency states. As a result, definition of threshold values for commencement of intervention and successful treatment are vital. Preferably, reference ranges obtained from the general, healthy population living in the same area should be used to define these thresholds. Published data show that reference ranges for retinol, α-tocopherol, β-carotene and lycopene have been obtained for different populations including the Swiss,[13] Dutch,[14] Hispanic Americans,[15] Japanese,[16] Americans,[17] Italians[18] and Spanish.[19] Our literature survey however, revealed that reference ranges for the above micronutrient antioxidants are not available for the Kuwaiti population.

The present study was designed to establish reference ranges for serum concentrations of retinol, α-tocopherol, β-carotene and lycopene in a randomly selected, healthy, adult population sample of male and female Kuwaitis.

Materials and methods

Subjects

Two hundred and sixty Kuwaitis, 159 men aged 18–58 years (mean 32.5 years) and 101 women aged 18–63 years (mean 33.3 years), were enrolled. They were non-smokers and had

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ordinary eating habits, with none on a special diet or taking vitamin supplements. The staple diet consisted of rice and meat (mainly mutton and chicken). Pasta was consumed less regularly than rice and bread was eaten less regularly than pasta. Vegetables were not very prominent but there was ample consumption of a variety of fruits. The following subjects were excluded: pregnant women, those with malabsorption, lipoproteinaemia, liver disorders and those taking vitamin supplements or drugs (cholestyramine, neomycin, mineral oil, antacid, cortisone, phenobarbitone, caffeine). Following overnight fasting, peripheral venous samples were taken into evacuated tubes and kept in the dark to clot. They were centrifuged and sera were separated and frozen at –80°C pending analysis of the micronutrient antioxidants and total cholesterol, which were carried out within 1 month. The procedures used were in accordance with the standards of the Ethical Committee of Clinical Investigation at Kuwait University.

Chemicals and standards

The HPLC-grade solvents used were methanol, acetonitrile, dichloromethane, hexane, which were purchased from BDH Chemicals, Dorset, UK. Other solvents and chemicals were of analytical quality. These were: ethanol (Ajax Chemicals, Auburn, NSW, Australia), benzene (Merck, Darmstadt, Germany) and butylated hydroxytoluene (BHT; Fluka Chemie AG, Buchs, Germany). The standards, consisting of all-*trans* retinol, dl-α-tocopherol, lycopene, β-carotene, and the internal standard D-α-tocopherol acetate, were from Sigma (St Louis, MO, USA).

Standard solution

The stock standards retinol (1 mmol/L), α-tocopherol (1 mmol/L) and β-carotene (0.074 mmol/L) were prepared in absolute ethanol, while lycopene (0.037 mmol/L) was prepared in 4% benzene ethanol. All ethanolic solvents contained 0.05% BHT, which stabilised the micronutrient antioxidants. Working standard solutions were prepared by suitably diluting the appropriate stocks with an ethanolic solution of 50 μmol/L α-tocopherol acetate to give 0.5–8 μmol/L retinol, 5–50 μmol/L α-tocopherol and 0–4 μmol/L each of β-carotene and lycopene. All standard solutions were kept at – 20°C.

Sample preparation

The HPLC method used was the method of Thurnham *et al.*,[20] with modifications. Sample preparation was carried out under dimmed natural lighting with the exclusion of fluorescent light and direct sunlight at all times.

Briefly, ethanol (150 μL) containing 0.05% BHT was vortex-mixed with serum (150 μL) for 30 s to precipitate protein. Hexane (300 μL) was added followed by vortex-mixing for 60 s and centrifugation at 6000 r.p.m. for 5 min. The organic phase (150 μL) was removed and evaporated under nitrogen atmosphere at 40°C. Extracts were stored in an iced, insulated box ready for injection into the HPLC column.

Liquid chromatograph conditions

The liquid chromatographic system was from Shimadzu (Kyoto, Japan) and consisted of:

i. an LC-10AD pump for isocratic elution at a flow rate of 1.0 mL/min from mobile phase, acetonitrile/dichloromethane/methanol (12/44/44), by volume.
ii. a Shim-pack CLC-reversed-phase column 15 cm long × 6 mm internal diameter packed with octadecyl (C18) bonded to spherical silica (5 μmol/L particle diameter and 100 Å pore diameter) for separation.
iii. an SCL-10 A system controller for time programming and for setting wavelengths.
iv. a one-channel SP-10 AV ultraviolet–visible variable wavelength detector linked to a C-R5A Chromatopac integrator and printout system.

Analyses of micronutrient antioxidants

The HPLC was calibrated with working standards of retinol and α-tocopherol. A 150 μL aliquot of the mobile phase was added to reconstitute the dried down extract. A 30 μL aliquot of the reconstituted extract was injected into the HPLC system for concurrent analysis of retinol and α-tocopherol. Analyses were done in duplicate. At the end of the retinol and α-tocopherol run, the liquid chromatograph was equilibrated to base line before reprogramming for carotenoid measurements and calibration with working standards of β-carotene and lycopene. A further 30 μL of reconstituted extract was injected for concurrent analysis of β-carotene and lycopene. Analyses were done in duplicate.

Retinol, α-tocopherol and internal standard were detected at wavelengths of 325 nm, 292 nm and 285 nm, respectively; while β-carotene and lycopene were detected at 450 nm. The average retention times for retinol, α-tocopherol, internal standard, lycopene and β-carotene were 2.8, 3.9, 4.4, 4.1 and 5.3 min, respectively. Standard curves were evaluated by linear regression analysis based on the internal standard calibration, and were obtained by plotting peak-area ratios against the concentrations of the external standards.

Assay of total cholesterol

Plasma total cholesterol concentrations were assayed by a routine enzymatic method with a Dade Behring Dimension clinical chemistry analyser (Dade Behring International, Newark, USA).

Precision and accuracy

The precision of cholesterol assay was monitored by means of commercial control sera (Notrol and Abtrol, SA Scientific, San Antonio, TX, USA) with a preassayed value for cholesterol. Another control serum (US National Institute of Standards, Gaithersburg, MD, USA), also with designated values, was used for quality control of the micronutrient antioxidant assays. Analyses of quality control materials for the concentrations of retinol, α-tocopherol, β-carotene, lycopene and total cholesterol were performed repeatedly to obtain intra-batch coefficients of variation (CVs). The intra-batch CVs were 2.8, 1.9, 3.1, 3.0 and 1.5% for retinol, α-tocopherol,

β-carotene, lycopene and cholesterol, respectively. Inter-batch CVs obtained by analysing the control materials for 30 sequential days were 3.5, 2.7, 4.2, 3.3 and 2.0% for retinol, α-tocopherol, β-carotene, lycopene and cholesterol, respectively.

Percent recovery was performed on plasma samples spiked with known concentrations of each micronutrient, antioxidant and cholesterol. The mean percent recoveries of retinol, α-tocopherol, β-carotene, lycopene and cholesterol were 99, 97, 91, 90 and 99% respectively.

Statistical analysis

Statistical analyses were performed using SPSS 10.0 for Window software (SPSS, Chicago, IL, USA). The histograms of the micronutrient antioxidant data had logarithmic normal distributions that were skewed to the right. The data were log-transformed to allow parametric tests to be carried out. Differences in mean values between groups were evaluated by one-way analysis of variance and Student's *t*-test. Two-tailed *P*-values were used and statistical significance was considered at $P < 0.05$. Linear regression analysis was used to determine micronutrient antioxidant associations and their relationships with cholesterol and with age. Data are expressed as mean ± standard error of the mean (SEM) and 95% confidence intervals (CI).

Results

The micronutrient antioxidant concentration and α-tocopherol: cholesterol ratio of sera in the Kuwaiti population sample are summarised in Table 1. Retinol concentrations were similar in men and women. The α-tocopherol concentration and α-tocopherol:cholesterol ratio were significantly lower in women ($P < 0.0001$) than in men. The β-carotene, lycopene and cholesterol concentrations were significantly higher in women ($P < 0.0001$) compared to men.

Table 2 shows the mean values of micronutrient antioxidants obtained in this study and in studies from different countries. The serum retinol value for Kuwaitis was similar to the Spanish value but was lower than the values reported for other countries. α-Tocopherol values reported for other countries were higher than the value for Kuwaitis. The β-carotene value was higher in Kuwaitis than in American, Japanese and Spanish subjects, but lower than the values reported for Italian and Swiss subjects. The lycopene level was much higher in Kuwaitis than in the other populations.

The effect of age on serum micronutrient antioxidants is presented in Table 3. The younger subjects (aged ≤ 30 years) had a significantly lower serum concentration of α-tocopherol ($P < 0.0001$) and α-tocopherol : cholesterol ratio ($P < 0.0001$), and significantly higher lycopene concentrations ($P = 0.002$) than the older subjects (aged > 30).

Table 4 depicts the relationship between serum micronutrient antioxidants and total cholesterol. α-Tocopherol showed the highest correlation with cholesterol. Retinol, β-carotene and lycopene were also positively correlated with cholesterol, but to a lesser degree.

The associations of the micronutrient antioxidants are presented in Table 5. Retinol and α-tocopherol were significantly positively correlated with each other ($P < 0.0001$). The highest positive association of micronutrient antioxidants was between β-carotene and lycopene. Significant negative associations were shown between α-tocopherol and β-carotene, and α-tocopherol and lycopene.

Discussion

In this study, the first of its kind in the Arabian Gulf, we have reported the serum micronutrient antioxidant concentrations of a randomly selected population sample of male and female adult Kuwaitis. This study is a biochemical study and did not include dietary surveys. Methods for dietary surveys are, in general, less accurate than biochemical methods. Dietary surveys providing data on nutrient intake have to rely on food composition tables and sometimes the nutrient content in the tables does not correspond to the actual amount consumed. Also, in food tables the nutrient values are generally for raw and individual foods and, on the whole, do not

Table 1. Mean ± SEM and 95% CI values for serum micronutrient antioxidants in an adult Kuwaiti population

Analysis	Total	Men (n = 260)	Women (n = 159)	P-value (n = 101)
Retinol (μmol/L)	1.76 ± 0.02	1.77 ± 0.03	1.74 ± 0.04	0.570
95% CI	1.71–1.84	1.71–1.85	1.65–1.82	
α-Tocopherol (μmol/L)	20.0 ± 0.5	21.3 ± 0.6	17.3 ± 0.5	< 0.0001
95% CI	19.1–20.9	20.0–22.6	16.7–19.0	
Cholesterol (μmol/L)	4.1 ± 0.1	4.0 ± 0.1	4.3 ± 0.1	< 0.0001*
95% CI	4.0–4.2	3.8–4.1	4.2–4.5	
α-Tocopherol (μmol/L)/ cholesterol (mmol/L)	5.1 ± 0.1	5.6 ± 0.2	4.3 ± 0.1	< 0.0001
95% CI	4.8–5.3	5.2–5.8	4.0–4.6	
β-Carotene (μmol/L)	0.52 ± 0.03	0.29 ± 0.03	0.88 ± 0.05	< 0.0001*
95% CI	0.46–0.57	0.25–0.33	0.78–0.97	
Lycopene (μmol/L)	0.95 ± 0.05	0.71 ± 0.04	1.31 ± 0.10	< 0.0001*
95% CI	0.85–1.05	0.62–0.80	1.11–1.50	

P-values < 0.05 were considered significant. * Significantly higher in women than in men.

Table 2. Mean values of serum micronutrient antioxidants for different populations

Population	Age (years)	Retinol (μmol/L)	α-Tocopherol (μmol/L)	β-Carotene (μmol/L)	Lycopene (μmol/L)	Reference
Kuwaiti	18–63	1.76	20.0	0.52	0.95	this study
Swiss	35+	2.09	29.3	0.70	NA	(13)
Dutch	18–64	2.00	NA	NA	NA	(14)
Hispanic	4–74	NA	29.2	NA	NA	(15)
Japanese	7–70	2.60	24.4	0.50	0.42	(16)
American	4–93	1.91	25.7	0.34	0.40	(17)
Italian	14–75	2.09	26.2	0.66	0.56	(18)
Spanish	5–79	1.70	28.2	0.25	0.36	(19)

NA = not assessed.

Table 3. Effect of age on serum micronutrient antioxidant concentrations in an adult Kuwaiti population

Micronutrient antioxidant	≤ 30 years (n = 129)	> 30 years (n = 131)	P-value
Retinol (μmol/L)	1.75 ± 0.05	1.71 ± 0.05	0.538
α-Tocopherol (μmol/L)	18.1 ± 0.7	23.3 ± 0.9	< 0.0001
β-Carotene (μmol/L)	0.52 ± 0.05	0.49 ± 0.06	0.700
Lycopene (μmol/L)	1.10 ± 0.08	0.78 ± 0.06	0.002
α-Tocopherol (μmol/L): cholesterol (mmol/L)	4.7 ± 0.2	5.8 ± 0.2	< 0.0001

Data are expressed as mean ± SEM. Statistical significance was considered at $P < 0.005$.

Table 4. Relationships between serum micronutrient antioxidants and total cholesterol in an adult Kuwaiti population

Micronutrient antioxidant	Correlation coefficient (r)	P-value
α-Tocopherol	0.492	< 0.0001
β-Carotene	0.237	0.003
Retinol	0.234	0.003
Lycopene	0.229	0.004

Statistical significance was considered at $P < 0.05$.

Table 5. Association of serum micronutrient antioxidants

Micronutrients	Correlation coefficient (r)	P-value
Lycopene versus β-carotene	0.744	< 0.0001
Retinol versus α-tocopherol	0.257	< 0.0001
Retinol versus β-carotene	0.112	0.072
Retinol versus lycopene	0.037	0.552
α-Tocopherol versus β-carotene	–0.285	< 0.0001
α-Tocopherol versus lycopene	–0.316	< 0.0001

Statistical significance was considered at $P < 0.05$.

take into consideration nutrient/nutrient interactions, heating losses and bioavailability. By oral questionnaire and observation, we noted that the staple diets of the Kuwaitis were grains (predominantly rice), meat (mainly mutton and chicken), prawns and fish. Tomatoes, tropical fruits (e.g., citrus, banana, mango and guava), Mediterranean fruits (e.g., grapes, melons, pomegranates, figs), Kiwi fruit, dates and a variety of nuts (e.g., peanuts, almonds, pistachios, cashew nuts) are abundantly available and largely consumed by Kuwaitis.

For a reliable micronutrient data comparison, it is essential that the other studies were comparable to ours in that they also employed HPLC methods for micronutrient analyses. Strict quality control measures were instituted in the present study to guarantee reliability of our results, in order to compare them with previous studies. Also considered were the normal threshold values, which were retinol, ≥ 0.70 μmol/L;[21] α-tocopherol, ≥ 11.6 μmol/L;[22] and total carotenoids, 0.75 μmol/L.[23] These were found to be less than the mean micronutrient values for our Kuwaiti subjects (Table 1). As shown by comparison with studies done in other populations (Table 2), the mean concentration of serum retinol for Kuwaitis was lower than the values reported for Swiss,[13] Dutch,[14] Japanese,[16] American[17] and Italian[18] populations. We observed no sex-related differences in serum

retinol concentrations, which contrasts with some studies[16,18,19] but confirms the report by Winklhofer-Roob and colleagues.[24] Another discrepancy was seen in the serum mean for α-tocopherol, which was lower in Kuwaitis than in other populations[13–19] (Table 2).

In the present study, it was observed that sex was a significant determinant of serum micronutrient antioxidant concentrations. The finding of a significantly lower concentration of α-tocopherol in women than in men is discordant with the report by Ito *et al.*[16] for the Japanese study. Also in the present study, serum concentrations of both β-carotene and lycopene were significantly higher in women than in men (Table 1). In the Japanese[16] and Spanish studies,[19] significantly higher concentrations of serum β-carotene were reported in women relative to men.

Another significant factor for the serum micronutrient antioxidant concentrations of the Kuwaiti sample was age. The α-tocopherol concentration and α-tocopherol : cholesterol ratio were significantly lower while lycopene concentration was significantly higher in subjects aged ≤ 30 years, compared with older subjects (Table 3). Ito *et al.*[16] found increased serum β-carotene concentrations in persons aged 20–49 years. With increasing age, this was only maintained in female subjects. In that study a total population sample of 1814 subjects (618 men and 1196 women), whose ages ranged from 7 to 86 years, was studied. Because we sampled a small population, we could only compare the concentrations of the micronutrient antioxidants of two age groups (Table 3). Other studies[25,26] reported lycopene as the major carotenoid in younger subjects. It has been shown elsewhere that age is inversely related to plasma lycopene concentration.[27] Also, some studies demonstrate a direct association between age and serum β-carotene concentration.[28,29]

As expected and in keeping with their lipophilic property, we observed that all four micronutrient antioxidants that we studied were significantly correlated with serum total cholesterol. Furthermore, α-tocopherol showed the greatest association with the lipid, an observation confirmed by the Swiss study of Winklhofer-Roob *et al.*[24] The lipophilic association has some relevance to the extent to which these micronutrient antioxidants protect low-density lipoproteins (LDL) against oxidation by free radicals.[30] Among the lipoproteins, LDL is the major cholesterol carrier from the hepatic site of synthesis and aggregation to tissue receptor sites. α-Tocopherol is more localised and concentrated than the other micronutrients, both in the hydrophobic interior of biological membranes and phospholipid 'coats' of plasma lipoproteins.[30] Biochemical investigations have clearly demonstrated the protective role of α-tocopherol against LDL oxidation.[31]

The rationale for measuring plasma cholesterol to determine α-tocopherol : cholesterol ratio is that plasma concentration of α-tocopherol (the major moiety of vitamin E) varies with serum lipid and thus requires lipid standardisation. The measurement of lipid-standardised plasma vitamin E gained clinical interest when it became evident that the suboptimal range (beyond overt vitamin E deficiency) might be associated with increased risk of chronic diseases such as cardiovascular disease and cancer.[32] Because α-tocopherol is highly correlated with cholesterol in plasma, it has been recommended that its ratio to cholesterol in plasma (expressed as μmol/L α-tocopherol/mmol/L cholesterol) be considered a better index of vitamin E nutriture and a more reliable expression of adequacy than α-tocopherol concentration (μmol/L) alone.[33] The ratio of serum α-tocopherol : total lipids is particularly valuable as an index of vitamin E status in situations such as liver disease where plasma lipid level may be elevated pathologically. Vitamin E deficiency may also be missed if the lipid level is not taken into account.[34] Alternatively, in those situations where serum lipid is unusually low, vitamin E deficiency may be overestimated.[35]

Retinol is suggested to protect LDL against free radical damage by scavenging free radicals, specifically peroxyl radical (ROO).[36] β-Carotene is believed by some to function as a radical-trapping lipid-soluble antioxidant by deactivating reactive oxygen species such as singlet oxygen ($^1O_2$) and free radicals.[37] As revealed in the present study (Table 1) and in agreement with a previous study,[38] lycopene, a straight chain isomer of β-carotene, is present in human plasma in greater concentrations than β-carotene. Lycopene is also said to exhibit the highest physical quenching rate constant with singlet oxygen.[38]

The demonstration that β-carotene and lycopene showed a strong positive association (Table 5) is in keeping with their isomeric relationship; lycopene is a straight chain isomer of β-carotene. Also worthy of note is that both carotenoids were negatively correlated with α-tocopherol, and α-tocopherol was strongly correlated with retinol. The positive associations suggests significant biological antioxidant cooperation or interdependence, and vice-versa for the negative associations.

In spite of the limitation imposed by number, we have demonstrated that serum concentrations of the four micronutrient antioxidants studied are, in most instances, specific to the Kuwaiti population and all but retinol are sex-dependent. In keeping with these findings, we have tentatively established a reference range for retinol that is not specific to sex and established sex-specific reference ranges for α-tocopherol, β-carotene and lycopene. To establish more credible reference ranges for these micronutrient antioxidants in serum, a larger study than the present one is warranted.

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