Lutein ester in serum after lutein supplementation in human subjects

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Lutein, one of the major carotenoids present in serum, is also widely consumed by most populations. For the purpose of testing the potential health benefits of several carotenoids, lutein was supplied as part of an intervention trial to test whether the consumption of these food constituents reduces oxidative damage to human tissue components. Lutein from a natural source (15 mg/d as mixed ester forms) was supplied for 4 months to eighteen non-smoking, apparently healthy volunteers (nine men, nine women) aged 25–45 years. The serum carotenoid profile was analysed at baseline and monthly thereafter. On average, lutein concentrations increased 5-fold after the first month of supplementation (mean 1·34 (range 0·6–3·34) µmol/l). On reviewing the results, in those volunteers whose lutein levels surpassed 1·05 µmol/l (fourteeen of seventeen), we tentatively identified lutein monopalmitate along with another unidentified ester (possibly from a monoketocarotenoid) in serum. Lutein levels returned to baseline values and ester forms were not present 3 months after supplementation was discontinued. Their concentrations did not correlate with, and represented less than 3 % of, lutein levels achieved in serum. They were observed before development of, and despite the presence of, carotenodermia. To our knowledge, this is the first time xanthophyll esters have been described in human serum. In view of the fact that xanthophyll esters have not been previously reported in serum and chylomicrons, it seems unlikely that these ester forms would be a reflection of the contents of the capsule. They may indicate a 'ceiling effect' on or saturation of the transport capacity for xanthophylls, and may have been re-esterified *in vivo* because of the unusual dietary conditions. The determination of the physiological importance of this finding will require further investigation, although neither haematological nor biochemical changes were detected.

Lutein: Carotenoids: Antioxidants

Lutein, a non-provitamin A xanthophyll, is one of the major carotenoids present in serum of most populations (Olmedilla *et al.* 1997*b*). It is predominantly transported in HDL and shows a specific distribution pattern in human tissues (Parker, 1996). Lutein, along with β-carotene, is one of the most widely distributed carotenoids in fruits and vegetables consumed by man.

Lutein shows a high antioxidant activity (Chopra & Thurnham, 1993; Packer, 1993), and *in vivo* oxidative metabolites have been described in human subjects after lutein supplementation (Khachik *et al.* 1995; Olmedilla *et al.* 1997*a*), indicating biological activity as a potential cancer-prevention agent (King *et al.* 1997). Data from epidemiological studies indicate a protective effect of dietary carotenoids against a variety of chronic diseases (Mayne, 1996) and, more specifically, lutein and zeaxanthin have been associated with a lower risk of eye disease in the elderly (Bunce, 1994; Mares-Perlman *et al.* 1995).

Lutein has not been widely used in intervention trials until now. The observations we present here were obtained from a clinical trial within the framework of project AIR2-CT93-0888 (Directorate-General XII, European Union) with the aim of testing whether 'the consumption of foods rich in carotenoids reduces oxidative damage to human tissue components'.

Subjects and methods

Eighteen Spanish control subjects (nine men and nine women) were assigned to receive lutein within the framework of a clinical trial involving volunteers from five European countries randomized to receive natural extracts of lutein, β-carotene, lycopene or placebo. Inclusion criteria were as follows: non-smokers, non-vegetarians, non-users of vitamin and/or mineral supplements, normal biochemical and haematological profiles, serum retinol greater than 1·05 µmol/l and serum α-tocopherol greater than 16·25 µmol/l. The baseline characteristics of the subjects are shown in Table 1.

The procedures used were in accordance with the Declaration of Helsinki and the ethical standards of the Ethical Committee of Clinical Investigation of Clínica

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Table 1. Baseline characteristics of subjects receiving lutein supplementation

(Mean values and standard deviations)

	Men (*n* 9)		Women (*n* 9)	
	Mean	SD	Mean	SD
Age (years)	37	5	39	6
BMI (kg/m^2)	26·6	2·6	23·7	3·8
Serum cholesterol (mmol/l)	5·64	0·93	5·30	0·91
Serum HDL-cholesterol (mmol/l)	1·55	0·59	1·94	0·36
Serum retinol (µmol/l)	1·85	0·14	1·61	0·24
Serum α-tocopherol (µmol/l)	31·1	9·3	30·4	7·0
Serum lutein (µmol/l)	0·30	0·12	0·30	0·11

Puerta de Hierro. Written informed consent was obtained from all volunteers.

The supplementation protocol was as follows: 100 mg D-α-tocopherol/d (weeks 0–4), 15 mg lutein/d (weeks 5–16) and lutein plus D-α-tocopherol (weeks 17–20). Only one man dropped out of the study. Compliance was assessed by counting pills and was over 97 % in both men and women.

Lutein from marigold flower extract was supplied by Quest International (Unilever, Vlaardingen, The Netherlands). Lutein monopalmitate, lutein diester and mixed ester standards were kindly supplied by Dr W. Stahl (Heinrich-Heine Universitat, Düsseldorf, Germany).

The capsules were analysed by HPLC (Olmedilla *et al.* 1997*b*), using a Spheri-5-ODS column and a mobile phase of acetonitrile–methanol (85 : 15, v/v) and acetonitrile–dichloromethane–methanol (70 : 20 : 10, by vol.) in gradient elution from 5 to 20 min at 1·8 ml/min, with carotenoid detection at 450 nm. The results showed several peaks with the chromatographic behaviour of diesters and mixed esters of lutein, and a small broad peak with retention time and spectrum of lutein monopalmitate. Saponification was carried out using methanolic KOH (50 g/l) at room temperature for 3 min in the presence of 0·1 M-pyrogallic acid as antioxidant and using retinyl acetate as internal standard. After saponification of the capsule, all-*trans*-lutein (approximately 12 mg), 13-/15-*cis*-lutein (approximately 3 mg) and 3·3 mg α-tocopherol were identified. Zeaxanthin was not detected.

Fasting blood samples for analysis of carotenoids, retinol, tocopherols, and biochemical and haematological profiles were taken at baseline, each month during the supplementation period and 3 months later. Serum samples were stored at −70° and extracted (1 ml) and analysed by HPLC (Olmedilla *et al.* 1997*b*) within 1 year. All samples from each volunteer were analysed on the same day. To further confirm the presence of these putative ester forms, we ran the samples using a second chromatographic system (Stahl *et al.* 1993) that consisted of a column of 5 µm Suplex PKB 100 eluted with methanol–acetonitrile–2-propanol (54 : 44 : 2, by vol.) and a flow rate of 1·5 ml/min, with detection at 450 nm.

Putative ester forms were identified by their chromatographic behaviour under the two systems, coelution of samples with lutein monopalmitate standard and comparison of spectral characteristics with those of authentic standards.

Eluting fractions corresponding to these putative ester forms were collected, both as a whole and separately, and saponified. Quantification of 'ester forms' was carried out using all-*trans*-lutein as standard ($A_{1 cm}^{1\%}$: 2550, ethanol at 445 nm) (De Ritter & Purcell, 1981).

Quality control of the analysis is routinely performed through the Quality Assurance Programme conducted by the National Institute of Standards and Technology (Gaithersburg, MD, USA).

The presence of carotenodermia was evaluated by the authors by visual examination of the palms of the volunteers and confirmed by an uninvolved physician (blind evaluation).

Statistical analysis was carried out by multifactor ANOVA to examine the effects of sex and time on plasma lutein concentration, assessing the interaction of these factors. Duncan's multiple range test was also performed.

Results

Lutein supplementation led to, on average, a 5-fold (range, 2- to 7-fold) increase in serum levels of lutein. This increment could be observed after the first month of supplementation (Olmedilla *et al.* 1997*a*) (mean 1·34 (range 0·6–3·34) µmol/l) (Table 2), and in most volunteers two small peaks eluted with retention times longer than that of retinyl palmitate (retention time 14 min according to the HPLC method described by Olmedilla *et al.* 1997*b*), and with a u.v.-visible spectrum that matched that of the lutein standard. These peaks (referred to as peaks 1 and 2 in Fig. 1) were detected in fourteen volunteers (nine women and five men) with lutein concentrations of 1·05 µmol/l or over, in most cases (eleven of fourteen), after the first month of supplementation. Both sexes behaved similarly and no significant changes were observed in total cholesterol, HDL-cholesterol or LDL-cholesterol levels at any time during the study.

Under the two different chromatographic systems (Stahl *et al.* 1993; Olmedilla *et al.* 1997*b*), the lutein

Table 2. Serum lutein concentrations of men and women supplemented with α-tocopherol and/or lutein for 20 weeks

(Mean values, median values and ranges for nine men and nine women)

Week	Men			Women		
	Mean	Median	Range	Mean	Median	Range
	Basal situation					
0	0·30	0·28	0·14–0·58	0·30	0·30	0·12–0·55
	α-Tocopherol (100 mg/d): weeks 0–4					
4	0·35	0·33	0·16–0·65	0·31	0·30	0·14–0·60
	Lutein (15 mg/d): weeks 5–16					
8	1·38	1·37***	0·60–2·59	1·55	1·28***	0·86–3·15
12	1·40	1·20***	0·56–3·06	1·68	1·53***	0·32–3·34
16	1·43	1·27***	0·53–3·08	1·58	1·46***	0·69–2·80
	α-Tocopherol + lutein: weeks 17–20					
20	1·35	1·13***	0·49–2·66	1·48	1·30***	0·77–2·45
	No supplementation: weeks 21–32					
32	0·27	0·26	0·18–0·35	0·29	0·26	0·16–0·49

Median values were significantly different from the corresponding values for weeks 0, 4 and 32, ***$P < 0.0001$.
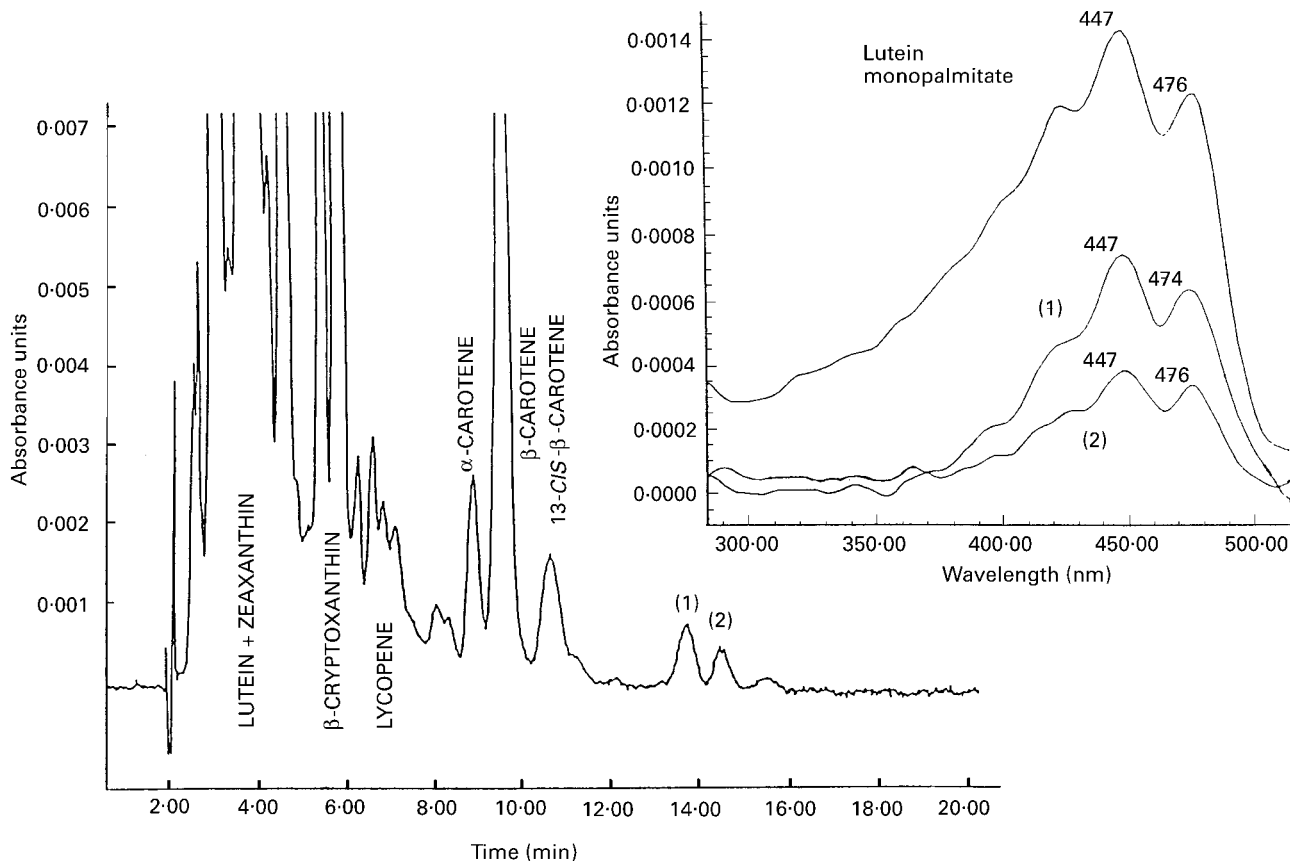
Fig. 1. Serum chromatogram of a volunteer who had received lutein supplementation for 1 month. In the box, spectra of: peak 1, peak 2 (lutein monopalmitate in serum) and lutein monopalmitate standard. Chromatographic conditions were according to Stahl *et al.* (1993) (flow rate: 1·5 ml/min; detection wavelength: 450 nm).

monopalmitate standard coeluted with peak 2 and showed spectral characteristics that matched those of peaks 1 and 2 (see box in Fig. 1).

These peaks were detectable only during the lutein supplementation period (lutein alone from week 8 to week 16, and lutein plus D-α-tocopherol from week 16 to week 20) but not at baseline (week 0) or after 1 month of D-α-tocopherol supplementation (week 4). In all cases, peak 1 showed a higher response than peak 2. These peaks were not present 3 months after supplementation had been discontinued (week 32). The volunteers assigned in the study to receive β-carotene (15 mg/d), lycopene (15 mg/d) or placebo were checked for the presence of these peaks but in no case were they observed in these individuals.

Peaks 1 and 2 were detected in serum before development of, and regardless of the presence of, carotenodermia, which was observed in seven out of seventeen volunteers after 4 months. On the other hand, the occurrence of carotenodermia in the β-carotene and lycopene-supplemented groups was not associated with the presence of xanthophyll esters.

The eluting fraction of peak 1 plus peak 2 was collected from the column, saponified and injected onto the column again. It showed a single peak, with the retention time and u.v.-visible spectrum of all-*trans*-lutein, while peaks 1 and 2 were not detected (Fig. 2). Separate collection and saponification of fractions from peaks 1 and 2 rendered no

peaks and lutein respectively. The lutein monopalmitate standard was also collected from the column and saponified under the same conditions, and showed a single peak of lutein. Recovery of lutein from the lutein monopalmitate standard after collection and saponification was over 90 %.

The presence of artifacts during sample handling was ruled out by collecting and saponifying the corresponding fraction (retention time from 12·5 to 15 min in the system of Stahl *et al.* 1993) from three types of samples used as blanks: (a) eluted mobile phase, (b) serum from a patient with a severe malabsorption syndrome (with no carotenoids), and (c) baseline serum from a volunteer who showed peaks 1 and 2 after lutein supplementation.

Concentrations of 'tentatively' identified lutein monopalmitate in serum (peak 2) ranged from 0·009 to 0·023 μmol/l. Peak 1 was present at concentrations of 0·009 to 0·040 μmol/l assuming the response factor to be that of lutein monopalmitate. In no case did the sum of the two peaks represent more than 3 % of the serum lutein concentration.

Discussion

To the best of our knowledge, this is the first time that xanthophyll ester forms have been detected in human serum. However, as other techniques such as nuclear magnetic resonance or liquid chromatography–mass
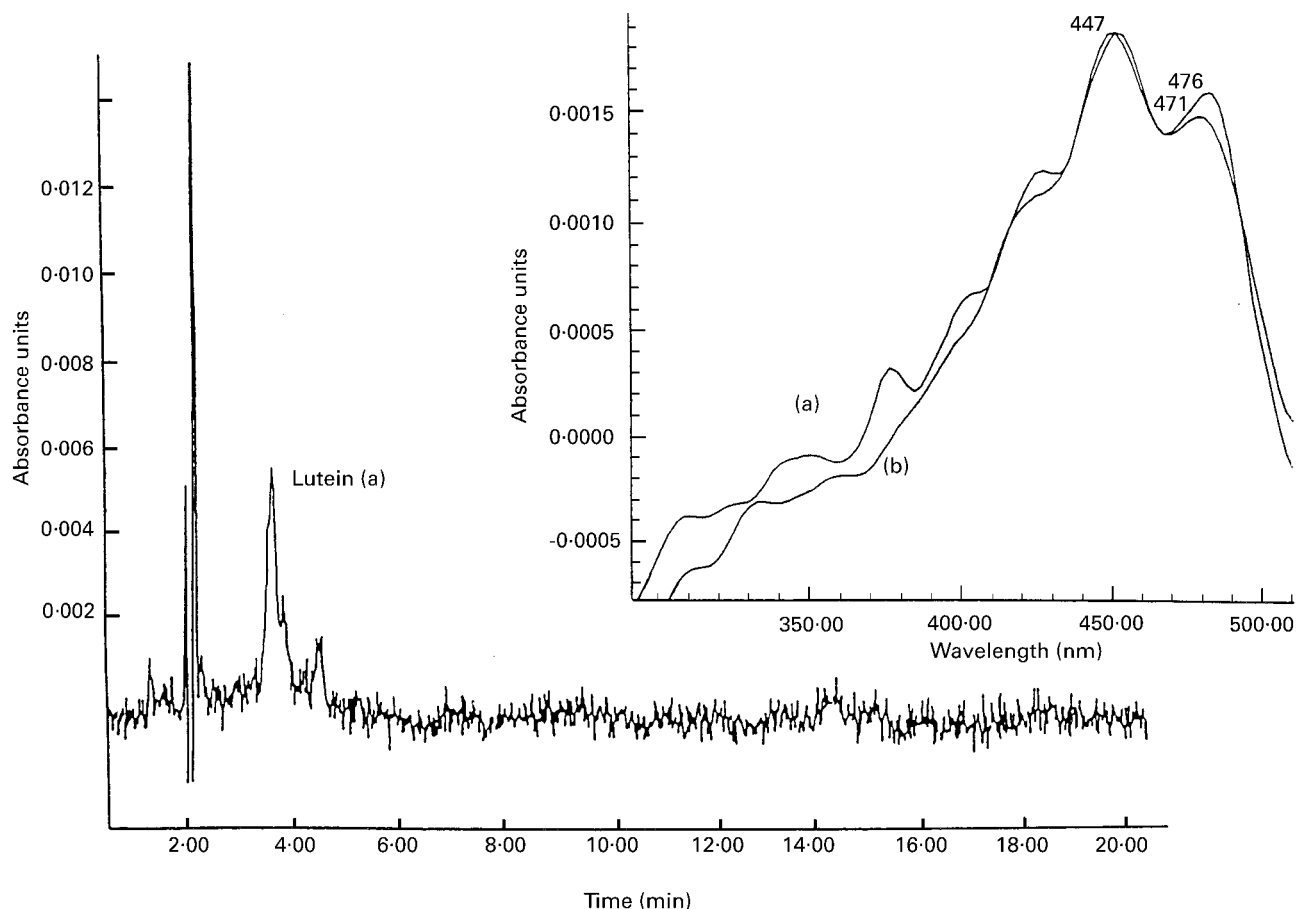
Fig. 2. Chromatogram of serum peak 2, collected and saponified. In the box, the spectra of lutein (a) from peak 2, and (b) from lutein monopalmitate standard, collected and saponified. Chromatographic conditions were as in Fig. 1.

spectrometry were not available, their identification should be considered to be tentative. Although lutein esters have been detected in skin (Dr W Stahl, personal communication), in our present study they were observed long before and regardless of the onset of carotenodermia. The presence of these ester forms was reversible and was associated exclusively with lutein supplementation. The absence of xanthophyll esters in serum (Khachik *et al.* 1995) and chylomicrons after ingestion of xanthophyll esters (Wingerath *et al.* 1995) is considered to indicate that, in man, these esters undergo cleavage before their release into the circulation (Wingerath *et al.* 1995). Moreover, the limited presence of lutein monoesters in marigold extracts (with palmitate, myristate and stearate together amounting to less than 7 %; Rivas, 1989) makes it unlikely, though nevertheless possible, that the serum content of this lutein monopalmitate came from the capsule, as it would have to have been absorbed selectively and intact.

The presence of these ester forms could also be due to an *in vivo* re-esterification process. Nethertheless, because of the design of the present study, no samples were collected postprandially (chylomicrons) and, thus, their origin (intestinal or other tissues) could not be identified. Moreover, their presence may be related not only to the lutein concentration achieved but to the length of time this level is maintained.

Although the presence of lutein monopalmitate (peak 2)

in serum was tested under two chromatographic systems, the identity of peak 1 remains unclear, and it does not seem to be a lutein ester attached to a shorter fatty acid as no lutein was recovered after saponification. Lutein oxidation products, identified as monoketo-monohydroxy-carotenoids and diketocarotenoids (Khachik *et al.* 1995), have been reported to increase in plasma on ingestion of lutein and/or zeaxanthin (Khachik *et al.* 1995; Olmedilla *et al.* 1997*a*); thus, peak 1, with its shorter retention time, might correspond to a monoketo-monohydroxy-carotenoid ester. The fact that no carotenoid peaks were detected after saponification of peak 1 may be due to the lability of carotenoids under alkaline conditions and/or incomplete recovery during partition.

The occurrence of ester forms in subjects having a serum lutein concentration of over 1·05 µmol/l may indicate a saturation-level capacity to deal with lutein when it is supplied in excess of normal dietary intake. A concentration of 1·05 µmol/l is not normally found in the Spanish population (0·18–0·69 µmol/l) nor in other populations with different dietary habits (Olmedilla *et al.* 1997*b*). This effect would be specific for lutein (and lutein oxidative metabolites?) and might be related to its polarity and distribution in lipoproteins (Parker, 1996).

The presence of lutein esters does not seem to be related to total carotenoid concentrations, given that in volunteers

from the present study supplemented with β-carotene presented with higher concentrations of β-carotene (up to 4·3 µmol/l) and total carotenoids (up to 8·1 µmol/l), no ester forms were detected.

Although lutein is transported predominantly in HDL, lipoprotein fractionation was not carried out and, thus, whether these esters are of hepatic or extrahepatic origin remains unknown. Ester forms are more hydrophobic and their distribution may be different from that of free lutein in the lipoproteins, affecting transfer among lipoproteins (Parker, 1996) and, thus, bioavailability to tissues (i.e. retina). Finally, the possible physiological significance of these forms in serum requires further investigation, although neither haematological nor biochemical changes were observed in the present study.

Acknowledgements

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