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Improved normal-phase and reversed-phase gradient high-performance liquid chromatography procedures for the analysis of retinoids and carotenoids in human serum, plant and animal tissues ☆

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Abstract

Two high-performance liquid chromatography (HPLC) procedures, a rapid normal-phase isocratic method for the analysis primarily of retinol and retinoic acid on a 3 μ silica column, and a reversed-phase gradient method for the simultaneous analysis of retinoids and very polar to nonpolar carotenoids on a 3 μ C_{18} column, are described. The normal-phase isocratic HPLC procedure is rapid (12 min), requires a sample size of 100 μl or less of serum, and is suitable for routine analysis of retinol in any serum, and of retinol and retinoic acid in serum after administration of retinoic acid. The reversed-phase gradient method is suitable for the simultaneous analysis of very polar to nonpolar carotenoids such as epoxy-xanthophylls and xanthophyll esters, along with other carotenoids and retinoids that occur normally in human serum and other plant and animal tissues. A run time of 30–70 min is necessary, depending on the presence or absence of xanthophyll esters in the sample.

Keywords: Retinoids; Carotenoids; Xanthophyll epoxides; Xanthophyll esters; Retinol; Retinoic acid

1. Introduction

Retinol, retinyl esters, tocopherols, β-carotene, lutein, lycopene and β-cryptoxanthin are the predominant forms of lipid soluble compounds in human and animal blood and other animal and plant tissues; for reviews see [1,2]. Other retinoid metabolites such as retinoic acid, 4-oxo-retinoic acid and retinoyl β-glucuronide also occur, but are present in very low concentrations. In recent years, high-performance liquid chromatography (HPLC) has become the method of choice for the analysis of retinoids and carotenoids. Quite a large number of HPLC procedures have been reported for the HPLC analysis of retinoids and carotenoids on C_{18} columns [1]. The use of C_{30} stationary phase for the analysis of carotenoids in food by HPLC has been recently reviewed [3]. Most of the published procedures for simultaneous analysis of polar retinoids, such as retinoic acid, along with nonpolar retinoids such as retinyl esters, utilize reversed-phase (RP)-HPLC and solvent gradient resulting in much longer time for analysis, in addition to time required for equilibration between two analyses [1]. Normal-phase HPLC (NP-HPLC) of retinoids and carotenoids is not as popular as RP-HPLC, mainly because most of the published procedures allow analysis of a single retinoid, usually a mixture of geometric isomers, at a

☆ Dedicated to the memory of James Allen Olson

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time. There are only a few NP-HPLCs that allow simultaneous analysis of retinol and retinoic acid [4,5], but availability of internal standard [4], or use of multiple step gradient [5], or retention of other lipids in the column might limit use in large epidemiological surveys. In the first part of this paper, a rapid normal-phase isocratic HPLC procedure, which is a modification of the published procedure [4], is described for the separation and quantitation of retinol and retinoic acid using easily available acitretin as an internal standard.

In recent years, besides β-carotene, which is the best provitamin A carotenoid, other carotenoids without provitamin A activity have emerged as a class of important compounds for prevention of certain diseases. Epidemiological data tend to support an association between intake of lycopene-rich tomato-based foods and a lower risk of prostate cancer [6,7]. Similarly, an inverse association between the incidence of age-related macular degeneration and combined lutein–zeaxanthin intake in the diet and the lutein and zeaxanthin concentration in blood serum has been reported [8]. Unesterified lutein is present in significant concentrations in human serum [1,2,9–12], milk [12] and other tissues [10]. Unesterified lutein and zeaxanthin are the major carotenoids found in the macula of the eye [13,14]. Lutein, whether ingested as the nonesterified or esterified forms, seems to be well absorbed [15,16]. Most of the published HPLC procedures used for the analysis of serum and tissue samples are not able to elute xanthophyll esters which are retained on the column [17]. Due to the lack of analytical methods, however, it is not known whether lutein esters are absorbed intact, or must be hydrolyzed to lutein before absorption can take place. Recently, by use of new HPLC procedures, the presence of very small quantities of lutein esters in human skin [18] and plasma [19], after supplements with lutein esters, have been reported. Epidemiological studies show that an increased intake of fruits and vegetables is associated with decreased risk of some cancers [20] and cardiovascular disease [21]. Whether the observed association between consumption of fruits and vegetables and the incidence of cancer and other chronic diseases is due to β-carotene, or to some other carotenoids and/or phytochemicals is still not clear [2]. Epoxycarotenoids, which are widely distributed in nature, constitute major dietary carotenoids in a number of fruits and vegetables [22,23]. It is not known if epoxycarotenoids might have some role in prevention of disease. In two recent studies from this laboratory, β-carotene 5,6-epoxide was found both to be much more active than β-carotene in inducing the differentiation of NB4 cells [24], and to be absorbed well by humans [25]. Unlike the mono-epoxy-carotenes, epoxyxanthophylls such as violaxanthin, lutein epoxide and neoxanthin are more abundant in our diets and constitute as high as 30% of the total carotenoids in many common vegetables and fruits that humans consume daily [17]. However, it is not known if the epoxy-xanthophylls are absorbed by humans like the the monoepoxy-β-carotenes. Development of an analytical method for simultaneous analysis of very polar carotenoids such as neoxanthin, violaxanthin, lutein 5,6-epoxide and nonpolar carotenoids such as lutein esters and zeaxanthin esters, might, therefore, be useful to study if epoxy-xanthophylls and xanthophyll esters are absorbed from dietary sources. Such an analytical method also would provide information about any circulating xanthophyll esters, if any, in humans.

In the second part of this paper, a RP-gradient HPLC method that is able to analyze simultaneously very polar violaxanthin, lutein epoxide, lutein and least polar xanthophyll esters, along with other plasma carotenoids, retinoids and tocopherols, is described.

2. Materials and methods

All experiments were carried out in laboratories lighted with Gold fluorescent lights (F40 Gold).

2.1. Chemicals and solvents

Anhydrous diethyl ether, acetone, acetonitrile, hexane, methanol, dichloromethane, ethyl acetate and NaOH were purchased from Fisher Scientific, Fair Lawn, NJ, USA. Whenever available, HPLC-grade solvents were used for HPLC. 3-Chloroperoxybenzoic acid was purchased from Aldrich (Milwaukee, WI, USA). Neutral alumina (Brockman activity I) (Woelm Pharma, Eschwege, Germany) was deactivated with water (5%, w/v). Lutein (stan-

dard) was obtained from Kemin Industries (Des Moines, IA, USA.). All-*trans* retinol, α-tocopherol, and γ-tocopherol were purchased from Sigma, St Louis, MO, USA. β-Carotene, acitretin (internal standard) and zeaxanthin (standard) were gifts from Hoffmann La Roche, Basel, Switzerland.

The standard solution of acitretin for NP-HPLC analysis was prepared by dissolving acitretin (4 mg) in CH_2Cl_2 (2 ml) and then diluting with hexane until an optical density reading of 0.5–0.6 at λ_{max}=361 nm was obtained.

2.2. Thin-layer chromatography

Thin-layer chromatography (TLC) was carried out on precoated silica (0.25 mm) plates (20×20 cm) (Macherey–Nagel, distributed by Brinkmann Instruments, Westbury, NY, USA). A solvent mixture of hexane:acetone (4:1, v/v) was used. Developments were carried out in glass tanks at ambient temperature.

2.3. Spectrophotometry

UV-visible spectra were recorded on a Shimadzu model 2010 spectrophotometer. The concentrations of retinoids and carotenoids were determined using published extinction coefficient values [1,26].

2.4. HPLC analysis

Two sets of Waters Associates (Milford, MA, USA) HPLC equipment were used with similar results. The first set (Set 1) that included two pumps (Model 510), a photodiode array detector (Model 991), an automated gradient controller, and a printer plotter (Model 5200), was used with a NEC (Model Power-Mate SX plus) computer. The second set (Set 2) included two pumps (Model 510), a photodiode array detector (Model 996), gradient control module, a Millennium 2010 chromatography manager, an autosampler (WISP model 717 plus) and a Hewlett-Packard Laser Jet printer. When Set 1 was used, the samples were injected manually in a Rheodyne injector.

2.4.1. NP-isocratic HPLC

For normal or straight phase isocratic analysis, Rainin (Woburn, MA, USA) Microsorb 3 μ silica column (0.36×10 cm) was preceded by a guard column packed with the same material. The solvent system consisted of a mixture of hexane:2-propanol:acetic acid (100:0.5:0.1, v/v) at a flow-rate of 1.0 ml/min.

2.4.2. RP-gradient HPLC

For reversed-phase gradient analysis, a Rainin (Woburn, MA, USA) Microsorb 3 μ C_{18} column (0.36×10 cm) was preceded by a guard column packed with C_{18} material. A combination of isocratic and gradient programs, as described in Table 1, was primarily used. In some runs, isocratic elution with solvent B was continued at a flow-rate of 1.2 ml/min for 40 min. Thus, the total run time for one analysis ranged from 60 to 70 min. The gradient was reversed to original conditions in 5 min, and the column was allowed to equilibrate with solvent A for 10 min. Chromatograms (520 nm to 280 nm range) were stored in the software memory. Chromatograms were plotted at 445 nm and 400 nm for carotenoids, at 325 nm for retinoids and at 285 nm for the tocopherols.

2.5. Extraction of lutein from marigold flowers

2.5.1. Lutein esters

Lutein esters were extracted by a slight modi-

Table 1
Gradient program used for the separation of carotenoids, retinoids and tocopherols

Time (min)	Flow rate (ml/min)	Solvent A[a] (%)	Solvent B[b] (%)	Gradient type
0–5	0.6	100	0	Isocratic
5–25	0.6–1.2	0	100	Linear
25–60	1.2	0	100	Isocratic
60–65	0.6	100	0	Linear

[a] A: Acetonitrile:water [97.5:2.5 (v/v); containing 10 m*M* ammonium acetate].
[b] B: Acetonitrile:dichloromethane:water [70:30:1 (v/v) containing 10 m*M* ammonium acetate].

fication of commonly used procedures [27,28] as follows: Dry petals of locally grown dry marigold flowers (3 g) were ground to a powder, and the carotenoids were extracted with acetone. The extract was decanted and kept cold. The residue was extracted further with acetone until the powder was almost colorless. The pooled extract was filtered, and then evaporated to dryness in a rotary evaporator. The residue was dissolved in a few drops of acetone. Analysis of a test solution by TLC showed that the extract contained primarily lutein esters (R_F=0.99) and only a trace of free lutein (R_F=0.2).

2.5.2. Lutein

For the preparation of lutein, dry petals of marigold flowers (30 g) were treated with methanolic NaOH (10%, w/v) (100 ml), and the mixture was refluxed for 1 h. Acetone (100 ml) was added, and the mixture was kept at room temperature overnight. The extract was decanted, and the petals were extracted with acetone several times until the extracts were almost colorless. Water and hexane were added to the pooled extract to separate phases. The hexane phase was separated. The aqueous phase, which was yellow, was extracted twice or thrice with hexane until the yellow color was extracted. The pooled hexane extract was washed with water and then dried over anhydrous sodium sulfate. Solid lutein was obtained by removing the hexane in a rotary evaporator. Based on an E-value (1%, 1 cm) of 2550 in ethanol [1,26], the yield of lutein was 75 mg (2.5 mg/g dry petals). TLC on silica gel plates using a solvent mixture of hexane:acetone (4:1, v/v) showed only one spot of lutein whose R_F value (0.2) was the same as that of authentic lutein. HPLC analysis showed that authentic lutein and lutein extracted from marigold petals were identical. Lutein was also prepared by saponification of lutein esters as described above.

2.6. Extraction of carotenoids from mustard green leaves

Fresh mustard green leaves (30 g) were ground with acetone. Extraction with acetone was continued until the residue was colorless. Hexane was added, followed by water. The hexane phase was washed with water, dried over anhydrous Na_2SO_4 and then evaporated to dryness. The residue was dissolved in a mixture of CH_3CN:CH_2Cl_2 (3:2, v/v). An aliquot was analyzed by RP-HPLC.

2.7. Extraction of zeaxanthin esters from Gou Qi Zi berries

Zeaxanthin esters were extracted by the following commonly used procedure [27,29]: berries of *Gou Qi Zi* (10 g) were ground with a small quantity of water, then extracted first with acetone, then with hexane repeatedly until all the color was extracted. Water was added to separate the organic hexane phase. The upper hexane phase was removed and set aside. The lower aqueous phase was extracted several times until the hexane extract was colorless. The pooled hexane extract was dried over anhydrous Na_2SO_4. The dry extract was evaporated to dryness in a rotary evaporator, and the residue obtained as a bright red powder, was dissolved in a few drops of acetone. Analysis of the solution by TLC on a silica gel plate using hexane:acetone (4:1, v/v) as the solvent mixture showed that the extract contained primarily zeaxanthin esters (R_F=0.99) and only a trace of free zeaxanthin (R_F=0.2).

2.7.1. Zeaxanthin

For the preparation of zeaxanthin, zeaxanthin esters (50 mg) were saponified with methanolic NaOH (10%, w/v) (100 ml) in the same way as described for the preparation of lutein above. Solid zeaxanthin was obtained as a dark red powder. TLC showed only one spot of zeaxanthin (R_F=0.2). HPLC analysis showed that authentic zeaxanthin and zeaxanthin extracted from the berries were identical.

2.8. Preparation of lutein 5,6-epoxide and violaxanthin

The method of preparation of lutein 5,6-epoxide and violaxanthin was the same as that reported for the preparation of β-carotene 5,6-epoxide [25]. The preparation of lutein 5,6-epoxide is described herewith. Lutein (75 mg) was dissolved in diethyl ether (10 ml) and stirred for 2 h at room temperature with 3-chloroperoxybenzoic acid (75 mg). TLC analysis showed that most lutein had been converted to the epoxide. The solution was first washed with

aqueous NaOH solution (5%, w/v), and then with water. The ether phase was dried over anhydrous sodium sulfate, and then was evaporated to dryness. The residue was dissolved in the minimum volume of diethyl ether (~2 ml), and subjected to column chromatography on neutral alumina (2.5×25 cm). The column was developed first with hexane, and then with increasing volumes of diethyl ether in hexane (5–30%). Fractions of 5 ml were collected. Lutein was eluted first with 10–15% ether, followed closely by 5,6-epoxylutein (20–30% ether). Based on an *E* (1%, 1 cm)=2800 in ethanol [26], the yield of lutein 5,6-epoxide was 56 mg. The identity of lutein 5,6-epoxide (λ_{max}=472, 443, 420 nm) was confirmed by treatment with a trace of dilute HCl which converted the 5,6-epoxide quantitatively to the 5,8-furanoid compound (λ_{max}=448, 420, 390 nm) [26]. Lutein (t_R=12.2′; λ_{max}=475, 447, 422 nm in the HPLC solvent mixture) separated very well from lutein 5,6-epoxide (t_R=8.6′; λ_{max}=472, 443, 420 nm in HPLC solvent mixture) and from lutein 5,8-epoxide (t_R=8.8′; λ_{max}=448, 420, 395 nm in HPLC solvent mixture).

Violaxanthin was prepared in the same way as lutein 5,6-epoxide by reaction of zeaxanthin with 3-chloroperoxybenzoic acid. The reaction was stopped, as judged from TLC, when most of the zeaxanthin and initially formed antheraxanthin were converted to violaxanthin. Violaxanthin was purified by crystallization. Pure violaxanthin showed λ_{max}= 470, 438, 405 nm in methanol [26]. With a trace of HCl, violaxanthin was converted to auroxanthin, λ_{max}=425, 400, 375 nm. Violaxanthin (t_R=6.7 min) separated very well from antheraxanthin (t_R=10.2 min) and zeaxanthin (t_R=13.9 min). Violaxanthin thus prepared coeluted with violaxanthin isolated from mustard green leaves.

2.9. Extraction of retinoids and carotenoids from serum for analysis of retinol and retinoic acid by NP-HPLC

Human serum (100 μl) obtained from volunteers 1–3 h after receiving an oral dose of all-*trans* retinoic acid (50 mg/person) [31] was treated with absolute ethanol (200 μl), acitretin solution (20 μl) and HPLC solvent mix (hexane:2-propanol:acetic acid 100:0.5:0.1, v/v). The mixture was vortexed (30 s), and centrifuged (30 s, 1200 *g*). The upper layer was removed and kept cold in ice. The aqueous layer was vortexed with ethyl acetate (0.5 ml), and centrifuged. The upper layer was added to the previous extract. The aqueous layer was extracted once more with HPLC solvent mix, vortexed and centrifuged. The upper layer was added to the previous extracts. The pooled extract was evaporated to dryness in a stream of argon. The residue was dissolved in the HPLC solvent mixture (hexane:2-propanol:acetic acid; 100:0.5:0.1, v/v)(35 μl). An aliquot of 25 μl was injected.

2.10. Extraction of carotenoids, retinoids and tocopherols from serum for analysis by RP-HPLC

Extraction of carotenoids, retinoids and tocopherols was carried out by the procedure described previously [31] namely, serum (0.5 ml) was mixed with ethanol (1 ml) containing retinyl acetate (internal standard) and butylated hydroxytoluene (0.01%, w/v), ethyl acetate (1 ml) and hexane (1 ml). The mixture was vortexed (30 s), and then centrifuged (1 min at 1200 *g*). The supernatant solution was separated and kept cold. The pellet was vortexed and centrifuged as before with hexane (1 ml) twice. Water (0.5 ml) was added to the pooled supernatant solution, which was vortexed and centrifuged as before. The hexane phase was carefully pipetted into a glass tube and evaporated to dryness under a slow stream of argon. The residue was dissolved in a mixture of acetonitrile:dichloromethane (7:3, v/v) (100 μl). An aliquot of 50 μl was injected onto the HPLC system.

3. Results

3.1. NP-HPLC analysis of retinol and retinoic acid in human serum

Extraction of lipids in human serum obtained from human subjects 1 h after an oral dose of retinoic acid [31] with a mixture of hexane, ethyl acetate and 2-propanol in presence of acetic acid, followed by NP-HPLC analysis on a 3 μ silica column, revealed the presence of acidic compounds retinoic acid and bilirubin, along with retinol and carotenoids (Fig.

1B). The separation of retinoic acid (peak 4), acitretin (peak 5) and retinol (peak 7) in a standard mixture (Fig. 1A) and in a human serum (Fig. 1B) is shown. The retention times of bilirubin, retinoic acid, acitretin and retinol were 2.5, 3.8, 4.6 and 8.4 min, respectively, and their separation was very satisfactory.

In Fig. 2, the separation of retinoids, carotenoids and tocopherols present in the serum of a healthy human adult is shown. Chromatograms obtained at 285 nm for tocopherols, 325 nm for retinol, 350 nm for retinoic acid and 445 nm for carotenoids in serum are presented in Fig. 2A. The spectrum index plot of the retinoids in serum obtained at 350 nm is shown in Fig. 2B. The position of elution of endogenous retinoic acid (peak 4, Fig. 2B) which could not be quantitated, is shown by an arrow. It can be seen that the hydrocarbon carotenoids α- and β-carotenes, and

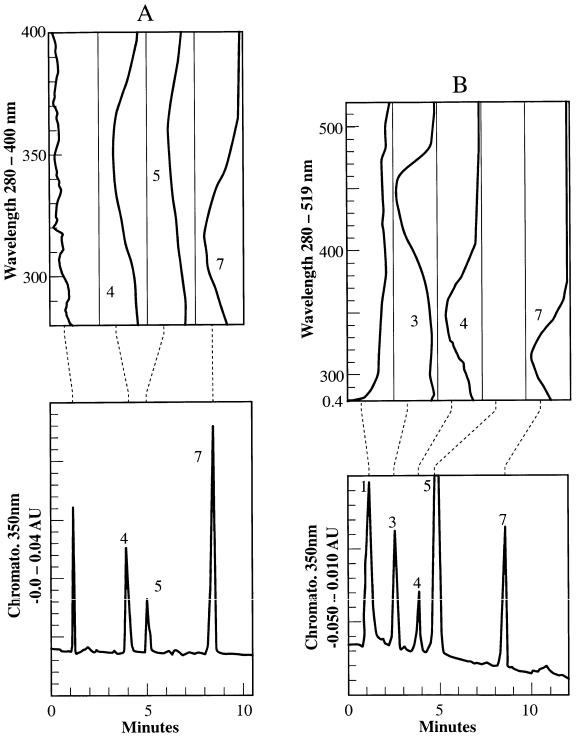

Fig. 1. Normal-phase isocratic HPLC analysis of retinoids in a standard solution and an extract of human serum 1 h after an oral dose of all-*trans* retinoic acid. Spectrum index plot at 350 nm of (A) standards and (B) serum extract. Peak identification 3: bilirubin; 4: all-*trans* retinoic acid; 5: acitretin (internal standard); 7: all-*trans* retinol.

lycopene did not separate, but eluted as a single peak (peak 1, Fig. 2B) with the solvent front. Similarly, lutein and traces of other dihydroxycarotenoids eluted together (peak 6, Fig. 1A). Continuation of development of the column for 30 min did not show the elution of any other compound that absorbed maximally at 285, 325, 350 or 445 nm.

It was found that all-*trans* retinoic acid (peak 4) and all-*trans* retinol (peak 7) separated well from their isomers, 13-*cis* retinoic acid (peak C4) and 13-*cis*-retinol (peak C7) as shown in Fig. 3.

3.1.1. Recovery and precision

When acetic acid was omitted during extraction of lipids, only traces of retinoic acid and bilirubin were detected. The recovery of acitretin which was used as the internal standard, was very poor (25–35%) in the absence of acetic acid. However, in presence of acetic acid, the recovery of acitretin in five replicate analysis was found to be 95±3%. The coefficients of variation (C.V.) in five replicate analysis of retinol and retinoic acid in a serum sample extracted in presence of acetic acid were 5 and 8%, respectively. The limit of detection and quantitation for retinoic acid was determined to be 0.4 ng and 0.6 ng, respectively, when detected at 350 nm. The detection and quantitation limits for retinol was 0.2 and 0.4 ng, respectively, when detected at 325 nm.

Furthermore, the concentrations of retinoic acid and retinol determined by the present procedure was within ±5% of the values obtained by a previous published procedure [30].

3.2. RP-HPLC analysis of lutein esters, lutein, lutein 5,6-epoxide, other carotenoids, retinoids and tocopherols

Analysis of marigold extracts, which primarily contained lutein esters, by HPLC on a Rainin column with a solvent gradient of methanol:water (7:3, v/v) containing 10 m*M* ammonium acetate to methanol:dichloromethane (4:1, v/v) [32] revealed that lutein esters did not elute even when isocratic elution with the latter solvent was continued for more than 1 h. Similarly, lutein esters could not be eluted by use of a solvent gradient of methanol:water (7:3, v/v, containing ammonium acetate) to acetonitrile:dichloromethane: methanol (95:10:5, v/v) [31]. There-

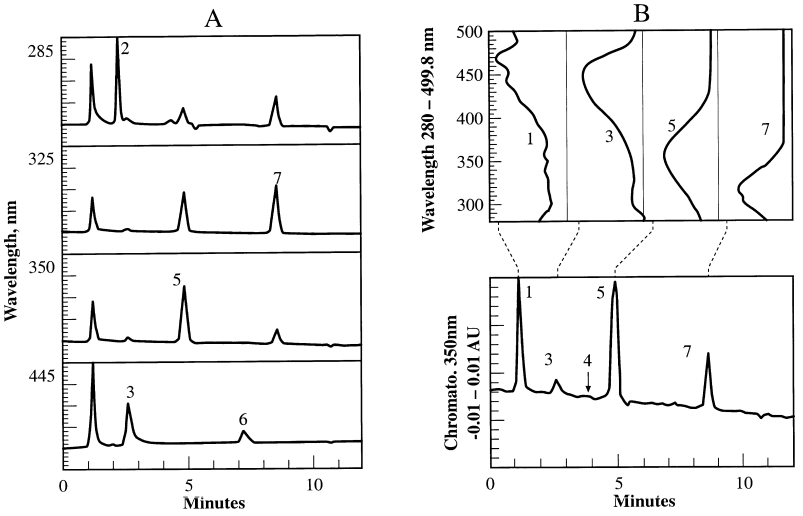

Fig. 2. Normal-phase isocratic HPLC analysis of retinoids in normal human serum. (A) Chromatograms of serum extract monitored at 285 nm (tocopherols), 325 nm (retinol), 350 nm (retinoic acid) and 445 nm (carotenoids). (B) Spectrum index plot at 350 nm of serum extract. Peak identification 2: tocopherols; 3: bilirubin; 4: all-*trans* retinoic acid; 5: acitretin; 6: lutein; 7: all-*trans* retinol.

fore, in the next experiment, an isocratic elution with a solvent mixture of acetonitrile:dichloromethane (7:3, v/v, containing ammonium acetate) at a flow-rate of 1 ml/min was tried on the Rainin C_{18} column. With this solvent mixture, four major lutein esters were eluted at 17.1, 22.6, 29.9 and 39.8 min. Traces of several earlier eluting lutein esters were also present. Thus, this method was found very suitable for analysis of lutein esters within 40 min. However, unesterified xanthophylls and lutein 5,6-epoxide, and other serum carotenoids were not well resolved by use of this solvent mixture.

A combined program of isocratic and gradient elution, as outlined in Table 1, however, resolved well a complex mixture of retinoids, tocopherols and carotenoids, including lutein, lutein 5,6-epoxide and lutein esters. As an example, chromatograms obtained with an extract of human plasma spiked with lutein 5,6-epoxide and marigold extract, mainly containing lutein esters, are shown in Fig. 4. The separation of γ-tocopherol (peak 1) and α-tocopherol (peak 2) is shown in Fig. 4A which was monitored at 285 nm, and the separation of retinol (peak 3) and retinyl acetate (internal standard; peak 4) is shown in Fig. 4B which was monitored at 325 nm. The chromatogram obtained at 445 nm for carotenoids is shown in Fig. 4C. The resolution of spiked lutein 5,6-epoxide (peak 5) from the major plasma carotenoid peaks (6: lutein; 8: 2′,3′-anhydrolutein, 10: β-cryptoxanthin, 11: lycopene and 13: β -carotene) is similar to that reported by others [14–16]. The major lutein ester peaks (14–17) separated very well. Minor peaks observed between β-carotene (peak 13) and the first lutein ester (peak 14), most of which showed the spectrum of lutein, are probably lutein monoesters as judged from their conversion to lutein on saponification.

3.3. RP-HPLC analysis of zeaxanthin esters

The application of the above RP-HPLC method for the analysis of extract of *Gou Qi Zi* berries showed the presence of a major zeaxanthin ester (t_R=60.6 min, peak 18, Fig. 5A), and a number of minor esters (t_R=21.5, 26.9, 31.8, 47.8 and 50.4 min). Based on published results [30], the major ester peak was dipalmityl zeaxanthin. Only a trace of unesterified zeaxanthin (t_R=13.9 min) was detected. Saponification of the extract followed by RP-HPLC analysis showed that all the minor and major peaks observed between 20 and 60 min disappeared, and a single peak of unesterified zeaxanthin was detected (peak 7, Fig. 5B).

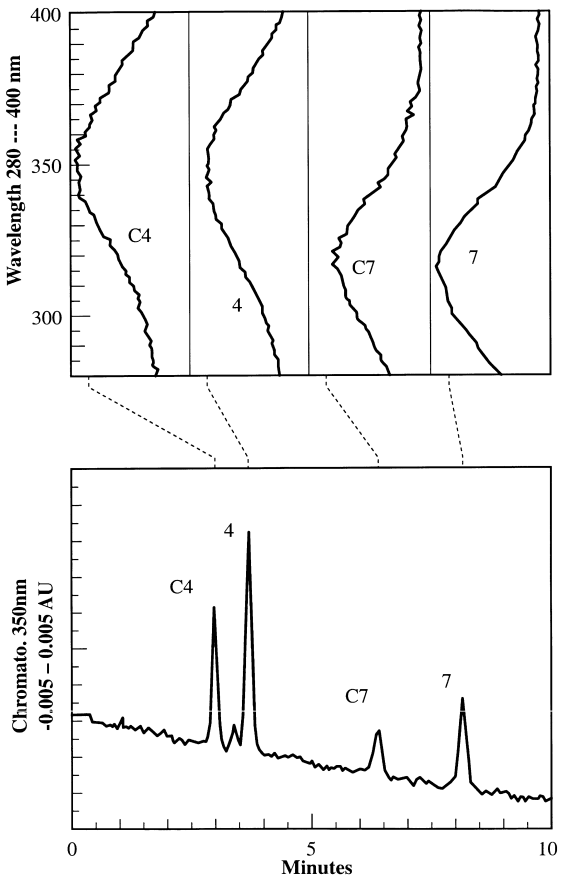

Fig. 3. Spectrum index plot obtained at 350 nm by normal-phase isocratic HPLC analysis of *cis-trans* isomers of retinol and retinoic acid. Peak identification C4: 13-*cis* retinoic acid; 4: all-*trans* retinoic acid; C7: 13-*cis* retinol; 7: all-*trans* retinol.

3.4. RP-HPLC analysis of carotenoid in mustard green leaves

Analysis of an extract of mustard green leaves showed that epoxy-xanthophylls, neoxanthin (peak N, t_R=5.4 min) and violaxanthin (peak V, t_R=6.7 min) which occur widely in a variety of green vegetables, separate very well from lutein (peak 6), β-carotene (peak 13) and chlorophylls (peaks CA and CB) (Fig. 6). No lutein esters were detected. Neoxanthin showed λ_{max}=465, 435, 410 and violaxanthin showed λ_{max}=470, 438, ~405 nm, in HPLC solvent. When the same extract was treated with a trace of diluted HCl, and an aliquot was analyzed by RP-HPLC, neoxanthin and violaxanthin were converted to neochrome and auroxanthin with λ_{max}= 445, 415, 390 and 425, 400, ~375 nm, respectively, due to conversion of 5,6-epoxy structure to 5,8-furanoid structure.

3.5. Recovery and precision

The recoveries of added retinyl acetate (internal standard) and spiked lutein 5,6-epoxide in serum samples in five replicate analysis were 96±5% and 94±3%, respectively. The coefficient of variation (C.V.) of β-carotene, lycopene and lutein were 8, 11 and 5%, respectively. The variation was greater (10–15%) for minor carotenoids, such as α- and β-carotenes.

4. Discussion

In the past, several RP-HPLC procedures for the simultaneous analysis of retinoids of different polarity, including retinol and retinoic acid, have been reported from this laboratory [30–33] and other laboratories; for a review see [1]. The published procedures allow very good separation of polar to nonpolar retinoids, but each analysis required 30–40 min of time. There are only a few normal-phase HPLC methods for simultaneous analysis of retinol and retinoic acid. The present method of normal-phase separation of serum retinoids described in this paper is an improved modification of the method by Meyer et al. [4]. The present method uses a 3 μ short column resulting in rapid (12 min) analysis of retinoic acid and retinol. Unlike the method of Lanvers et al. [5] which uses step gradient, no additional time was required for equilibration of the column between runs in the present method. Acitretin was used in the present study as an internal standard instead of ethyl sulfonic acid RO 15-1570 used by Meyer et al. [4]. Acitretin was found to be an excellent internal standard, and its recovery was satisfactory. Moreover, being a prescription drug for the treatment of psoriasis, acitretin should be more easily available. The serum samples used in this study were the same used in a previous study [30]. The samples were stored at −72°C. Comparison of the results for retinol and retinoic acid showed that the values were within ±5% error. It was also found

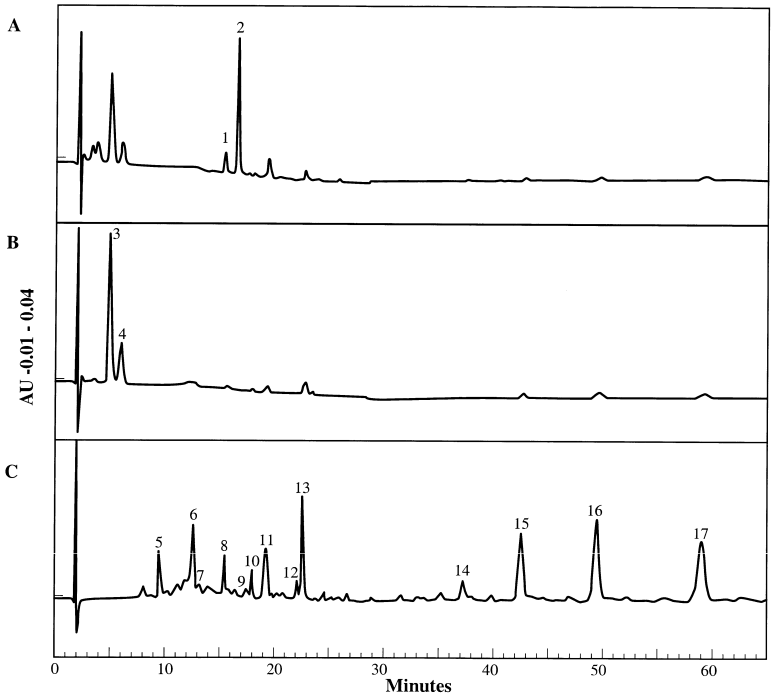

Fig. 4. Reversed-phase isocratic-gradient HPLC chromatograms of human serum spiked with lutein 5,6-epoxide and marigold extract. Chromatogram A for tocopherols was obtained at 285 nm; chromatogram B for retinoids was obtained at 330 nm, and chromatogram C for carotenoids was obtained at 445 nm. Peak identification 1: γ-tocopherol; 2: α-tocopherol; 3: retinol; 4: retinyl acetate (internal standard); 5: lutein 5,6-epoxide; 6: lutein; 7: zeaxanthin; 8: 2′,3′-anhydrolutein; 9: α-cryptoxanthin; 10: β-cryptoxanthin; 11: lycopene; 12: α-carotene; 13: β-carotene; 14–17: lutein esters.

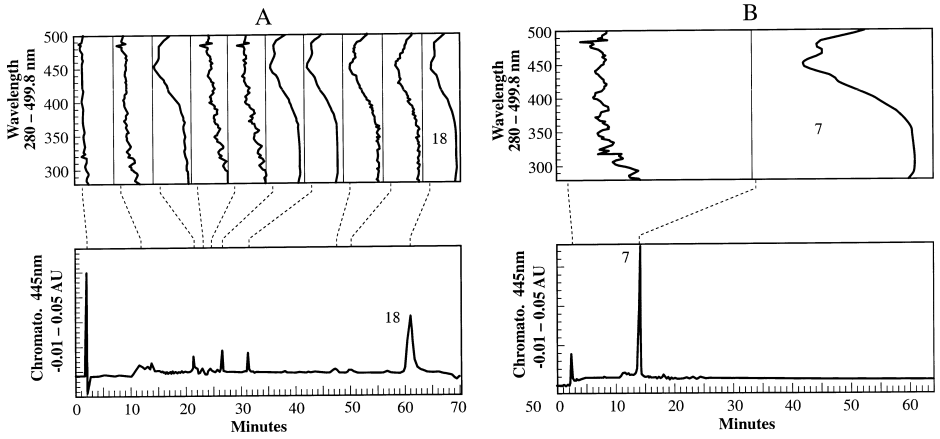

Fig. 5. Reversed-phase isocratic-gradient HPLC analysis of (A) zeaxanthin esters in berries of *Gou Qi Zi* and (B) zeaxanthin obtained by saponification of zeaxanthin esters. (A) Spectrum index plot obtained at 445 nm. Peak identification 7: zeaxanthin; 18: zeaxanthin ester.

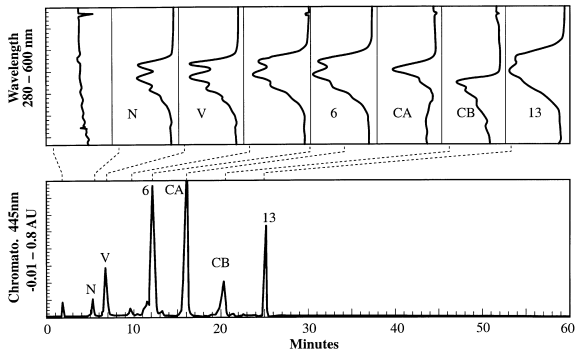

Fig. 6. Reversed-phase gradient HPLC analysis of carotenoids and chlorophylls in mustard green leaves. Peak identification N: neoxanthin; V: violaxanthin; 6: lutein; CA: chlorophyll A; CB: chlorophyll B; 13: β-carotene.

that the procedure was able to separate a standard mixture of *cis-trans* isomers of retinol and retinoic acid. However, applicability of the method for the separation of *cis-trans* isomers in human samples has not been tested. Endogenous retinoic acid could not be quantitated in the small serum samples of 100 μl that were used during this study (Fig. 2). However, the procedure described in this paper should be suitable for simultaneous analysis of retinoic acid and retinol in serum samples following a dose of retinoic acid. Because of the short analysis time, the method should be useful for analysis of retinol only in a large number of serum samples in epidemiological studies. Another advantage of the procedure is that all of the polar and nonpolar retinoids and carotenoids are eluted from the column during a single analysis, thus avoiding the danger of elution of polar compounds and pressure build-up during subsequent runs. As stated in the Results, it is very important to dissolve and inject the serum extract in the HPLC solvent mixture to obtain reproducible retention times. Presence of other solvents, such as dichloromethane, even in trace amounts, altered the retention times of retinoids; retinol in particular.

During NP-HPLC analysis of serum extracts, when the chromatograms were recorded at 445 nm for carotenoids, it was found that the hydrocarbon carotenoids, α-, β-carotenes and lycopene, coeluted as a single peak with the solvent front (peak 1, Fig. 2B). Two other carotenoid peaks, one carrying more polar lutein (peak 6, Fig. 2A), mixed with its isomers and zeaxanthin, and another peak carrying the monohydroxycarotenoids α-, β-cryptoxanthins (t_R=5.2 min, not shown) were also detected in other human sera.

The RP-gradient HPLC procedure described in this paper was found suitable for simultaneous analysis of a whole range of very polar to nonpolar carotenoids (xanthophyll epoxides, mono- and dihydroxyxanthophylls, hydrocarbon carotenoids, xanthophyll esters) and the retinoids, retinol and retinyl esters in particular. As demonstrated in Fig. 4, tocopherols separated well from other analytes, but analysis of tocopherols was not studied in detail. For simultaneous analysis of all the carotenoids, including xanthophyll esters, a run time of 70 min is necessary. However, if xanthophyll esters are not present in the sample, the analysis of other carotenoids, retinoids and tocopherols in subsequent samples can be completed in about 30 min time. Analysis of a large number of samples showed that the results were reproducible, and the column pressure was always low compared to other solvent mixtures.

The RP-gradient HPLC analysis has also been applied for analysis of carotenoids and retinoids in other animal tissues such as rat liver and intestine, and was found very satisfactory for analysis of retinyl esters as well (results not shown).

Rivas [28] found four major esters of lutein in marigold extracts; namely, the dipalmitate (37%), myristate–palmitate (24%), palmitate–stearate (15%), and dimyristate (11%) as well as smaller amounts of the distearate (4%) and a group of more polar, presumably monoesters of lutein (9%). The procedure reported in this paper, however, separated the lutein esters somewhat better than that of Rivas [28], although no attempts were made to identify the fatty acids present in each ester peak. Upon saponification, ''free'' lutein was by far the major carotenoid produced in this study as well as in others [34,35].

Lutein, together with a smaller amount of zeaxanthin, is a major carotenoid in the plasma of most well nourished humans [1,2,9–12] and is specifically found in the human macula [13,14]. Although it is presumed that dietary lutein esters are hydrolyzed to

free lutein in the intestine, absorbed as free lutein, and transferred to the plasma via the lymph [2], data are yet not available to conclusively support this presumption. It is not known with certainty whether ''free'' lutein is esterified, and then transported as lutein esters in the plasma. When lutein from a natural source (15 mg of mixed fatty acid esters/day) was fed to men and women subjects for 4 months, the five-fold increase in plasma lutein levels was accompanied by a small increment in plasma lutein esters (<3% of total plasma lutein) [19]. Small amounts (0.3–0.8 pmol/g net weight) of individual xanthophyll esters, including those of lutein, are also found in human skin [18]. The amounts present in skin, however, are <1% of the amount of β-carotene present in the skin [18]. The present improved HPLC method should allow a more detailed analysis of the formation and transport of xanthophyll esters than has previously been possible.

The identification of dipalmityl zeaxanthin as the major carotenoid in *Gou Qi Zi* berries has been reported [29]. Analysis of the berry extract by the present method confirmed that there was one major peak of dipalmityl zeaxanthin. The present method was superior to the published procedure, because it was possible to separate a number of other minor peaks which eluted between unesterified zeaxanthin and dipalmityl zeaxanthin. All these minor peaks disappeared upon saponification resulting in unesterified zeaxanthin only (Fig. 5). Thus, it was concluded that these minor peaks were presumably mono- and di-esters of zeaxanthin.

Carotenoid epoxides, although common constituents of fruits and vegetables, have not been studied much in a biological context in the past. β-Carotene 5,6-epoxide, however, enhances the differentiation of NB4 cells much better than β-carotene [24], is efficiently absorbed by humans [25] and gives rise to 5,6-epoxyretinyl palmitate in the plasma [25]. However, it is not known whether epoxy-xanthophylls such as neoxanthin, violaxanthin and lutein 5,6-epoxide, which are abundant in our diet, are also absorbed or not. Studies are in progress on the intestinal absorption of epoxy-xanthophylls and lutein esters in humans, and the RP-gradient HPLC procedure has been successfully used for the simultaneous analysis of epoxy-xanthophylls, xanthophyll esters and other commonly occurring carotenoids and retinol in human serum.

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