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Analytical Methods Section

Simultaneous analysis of individual carotenoids, retinol, retinyl esters, and tocopherols in serum by isocratic non-aqueous reversed-phase HPLC

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A sensitive high-performance reversed-phase liquid chromatographic assay which utilizes isocratic elution and detection by two detectors has been developed to measure simultaneously several carotenoids, retinol, retinyl esters, and α- and γ-tocopherols in serum. Although 100 μl of serum or plasma is used routinely, as little as 10 μl can be analyzed. Typical lower limits of detection (5 : 1 signal/noise ratio) are 0·2 pmol for retinol, 6 pmol for α-tocopherol, 1 pmol for β-carotene, and 2 pmol for retinyl palmitate. Although lutein and zeaxanthin were only partially resolved from each other, all other major serum carotenoids including α-carotene from β-carotene and α-cryptoxanthin from β-cryptoxanthin were completely separated. Retinyl hexanoate and β-apo-8'-carotenyl decanoate are convenient, readily prepared internal standards for this assay. Relative retention indices, based on elution times of alkylphenone standards, proved useful both for characterization of compounds and for comparison between columns.

INTRODUCTION

In both experimental and epidemiological studies, dietary carotenoids, retinoids and tocopherols have been implicated as possible protective agents against some types of cancer (Peto *et al.*, 1981; Willett & MacMahon, 1984; Ziegler, 1989; Greenberg *et al.*, 1990). HPLC procedures that deal with the analysis of retinol, tocopherols and carotenoids have been reviewed (De Leenheer *et al.*, 1988; Furr *et al.*, 1991).

In this paper, a new HPLC procedure is described, which allows the simultaneous separation and quantitation of all the major carotenoids, retinoids and tocopherols present in human serum. The assay has also been used to quantitate retinol and retinyl esters in mouse serum and liver.

METHODS

Reagents

Lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene were isolated from plant sources and purified by HPLC as described previously (Barua *et al.*, 1989). Synthetic lutein, lycopene, β-carotene and retinyl palmitate (Sigma Chemical Co., St. Louis, MO) were also purified by HPLC prior to use. Retinol was prepared by reduction of retinal (Sigma) with sodium borohydride (Hubbard *et al.*, 1971). β-Apo-8'-carotenol was prepared similarly by sodium borohydride-reduction of β-apo-8'-carotenol (Fluka, Ronkonkoma, NY). Esters of retinol and β-apo-8'-carotenol were prepared by reaction with the appropriate acyl anhydrides (Sigma) in triethylamine (Ross, 1981, 1986), followed by purification by HPLC as necessary. Alkylphenones were purchased commercially (Sigma; Aldrich Chemical Co., Milwaukee, WI) or synthesized by Friedel–Crafts reaction of benzene with appropriate acyl anhydrides (Furniss *et al.*, 1978).

Capacity factors and relative retention indices (retentions relative to alkylphenone standards; acetophenone = 800) were calculated as described previously (Furr, 1989) by using a homologous series of alkylphenones as standards.

HPLC grade acetonitrile and methanol and reagent grade dichloromethane, dichloroethane, and 1-octanol were obtained from Fisher Scientific Co. (Fairlawn, NJ).

High performance liquid chromatography (HPLC)

The HPLC system included a Waters model 510 pump and an automated injection system (WISP, Waters

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Associates, Milford, MA), connected in series to two ISCO (Lincoln, NE) model V4 detectors, one set at 450 nm (for carotenoids) and one set at 300 (for retinol and tocopherols) or 325 nm (for retinol and retinyl esters). A Waters C-18 5 μm 'Resolve' column (30 cm × 3·9 mm ID) was preceded by a C-130 guard column (Upchurch Scientific, Oak Harbor, WA). The detectors were connected to a dual-channel Shimadzu CR-4A integrator (Shimadzu Corp., Columbis, MD). Detector sensitivity was routinely set at 0·004 AUFS (attenuation 2) to detect carotenoids at 450 nm, and at 0·016 AUFS (attenuation 4) to detect retinol and tocopherols at 300 nm or at 325 nm to detect retinol and retinyl esters. The mobile phase for chromatography was acetonitrile/dichloromethane/methanol/l-octanol (90 : 15 : 10 : 0·1, v/v/v/v) which was filtered through a 0·45 μm nylon filter before use. The flow rate was 1·0 ml min $^{-1}$. Peaks were identified by comparing serum peak retention times relative to those of known standards. In several instances, individual peaks were identified by monitoring the UV absorbance spectra after the injection of concentrated extracts from about 1 ml of serum.

Extraction of carotenoids, retinoids and tocopherols from human serum

The extraction procedure was similar to the one reported previously (Barua *et al.*,1989). A solvent mixture of ethyl acetate and hexane, which was more efficient in extracting hydrocarbon carotenoids than hexane alone, was used. In brief, a solution of retinyl hexanoate (typically 10 μl of 13 μmol liter $^{-1}$, 5 ng μl $^{-1}$) and/or a solution of β-apo-8'-carotenyl decanoate (typically 10 μl of 5 μmol liter $^{-1}$, 3 ng μl $^{-1}$) in methanol was added to serum (100 μl). The serum was then treated with ethanol (200 μl) (containing either none or 0·01% (w/v) butylated hydroxytoluene (BHT)), and ethyl acetate (500 μl). The mixture was vortexed (30 s), and centrifuged (30 s × 2000 rpm). The supernatant solution was saved, and the pellet was broken and extracted with ethyl acetate (500 μl) twice, and then with hexane (500 μl). The mixture was vortexed and centrifuged at each step as just described. All the organic phases were pooled, water (500 μl) was added, vortexed and centrifuged as before. The upper organic phase was transferred carefully by means of a Pasteur pipette to a test tube, and the solvent was evaporated under a gentle stream of argon. The residue was dissolved in ice cold (to minimize loss due to evaporation) dichloromethane (40 μl). Methanol (60 μl) was added, and the solution was immediately transferred to a glass low-volume insert (Waters) that was placed inside a 1 ml WISP vial (Waters). The vial was capped, and put inside a Waters WISP autosampler. Aliquots of 2–50 μl were analyzed immediately. Low-volume inserts allow for a minimum sample waste volume of 6 μl.

When 20 μl of serum samples were used, the volumes of extracting solvents were reduced to 20% at each step. The final extract was dissolved in 50 μl of solvent mixture, and depending on single or duplicate analysis, 20–40 μl aliquots were injected.

Hypervitaminotic mouse sera and livers

Sera and livers of mice that had received supplements of retinyl acetate (1750 μg per mouse per day) for four weeks were analyzed for retinol and retinyl esters. Because no carotenoids were expected in mouse serum, a simpler extraction procedure was used as follows: to 50 μl of serum, 90 μl of 100% ethanol containing 0·01% BHT and 10 μl of retinyl hexanoate (104 μmol liter $^{-1}$, 40 μg ml $^{-1}$) in 100% ethanol was added and the sample was vortexed. The sample was then extracted twice with hexane (300 μl) and once with ethyl acetate (300 μl). The organic layers were pooled and solvent was evaporated to dryness under argon. The residue was dissolved in CH_3OH/CH_2Cl_2 (3 : 1, v/v; 50 μl); 45 μl of the sample was analyzed by HPLC. Livers were extracted according to Furr and Olson (1989), and 10–100 μl aliquots were injected.

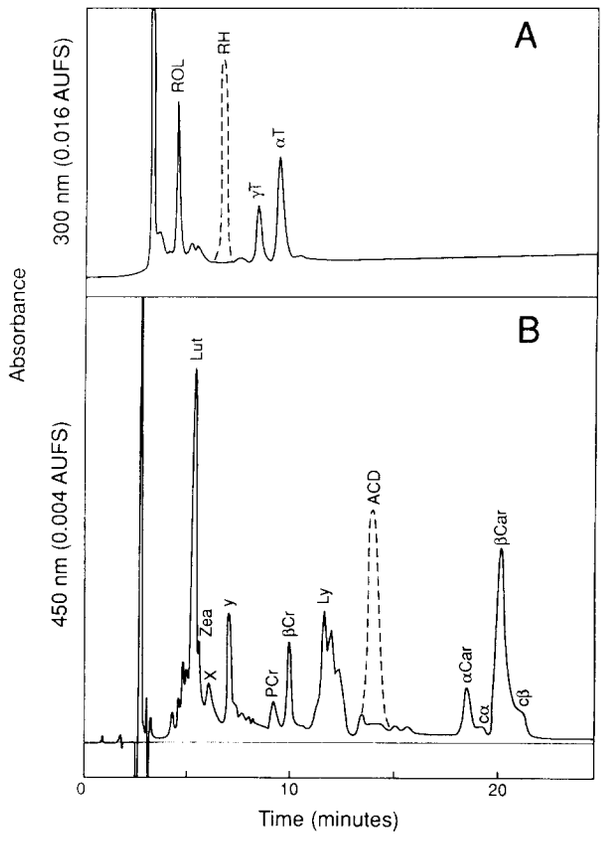

Fig. 1. Elution profiles of carotenoids (lower panel B), and retinol and tocopherols (upper panel A) present in 10 μl of human serum obtained in one assay by reversed-phase isocratic HPLC. Two detectors set at 450 nm (for carotenoids) and 300 nm (for retinol and tocopherols) were used in series. Peaks: Lut, lutein; Zea, zeaxanthin; X, unidentified (probably *cis*-lutein or *cis*-zeaxanthin); Y, unidentified (probably 2',3'-anhydrolutein); PCr, α-cryptoxanthin; βCr, β-cryptoxanthin; Ly, lycopene; ACD, apo-8'-β-carotenyl decanoate (internal standard); αCar, α-carotene; cα, *cis*-α-carotene; βCar, β-carotene; cβ, *cis*-β-carotene; ROL, retinol; RH, retinyl hexanoate (internal standard), γT, γ-tocopherol; αT, α-tocopherol.

RESULTS

Figure 1(B) shows the isocratic reversed-phase HPLC chromatogram obtained after injection of 10-μl extract of human serum (equivalent to 10 μl serum). Not only was a baseline separation of α- and β-carotenes achieved, but also *cis* isomers of the carotenes were partially resolved. Similarly, baseline separation of α-cryptoxanthin and β-cryptoxanthin was obtain. The unidentified carotenoids 'X' and 'Y' (Fig. 1(B)) also separated very well from each other. Lutein and zeaxanthin, however, were not completely resolved. With simultaneous detection at 300 nm, retinol and the tocopherols were also quantified (Fig. 1(A)).

After injection of 45 μl of hypervitaminotic mouse serum extract, retinyl esters (linoleate, palmitate, oleate, stearate) were well resolved (Fig. 2, upper panel). Retinyl esters in an extract of mouse liver were similarly resolved (Fig. 2, lower panel).

BHT was not used during the extraction of human serum (Fig. 1(A)). When included in sample preparations, however, BHT eluted just ahead of retinol (Fig. 2, upper panel). The tocopherols were not detected at 325 nm (Fig. 2, upper panel).

Limits of detection for this method and typical analyte ranges in human serum are presented in Table 1. The limits of detection are more than adequate for quantitation of all the listed analytes in 100 μl of serum or plasma. An aliquot of 10 μl of serum extract gave satisfactory results for carotenoids, retinol and tocopherols (Fig. 1), whereas 3 μl of serum extract was sufficient for quantitation of retinol and tocopherols.

For a human serum sample analyzed in quadruplicate (100 μl each) on three successive days, recovery of retinyl hexanoate averaged 98·7 ± 9·6%, and recovery of β-apo-8'-carotenyl decanoate averaged 95 ± 8·4%. Retinyl esters were not detected in this fasting blood sample (less than 20 nmol liter^{-1} retinyl palmitate).

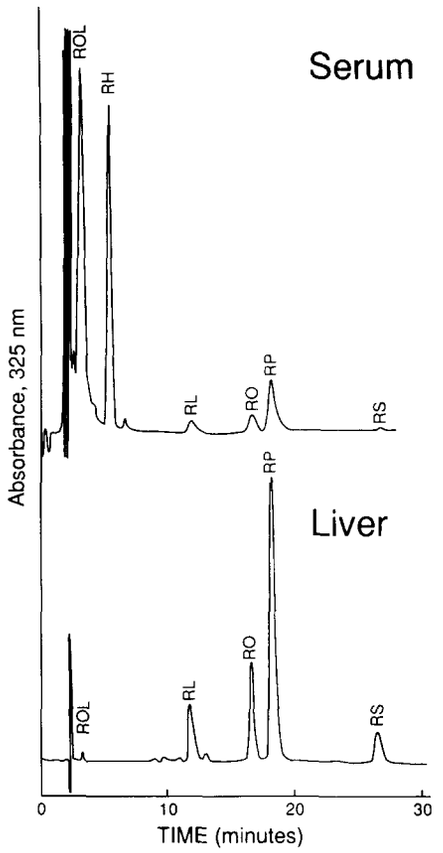

Fig. 2. Elution profiles of retinol and retinyl esters present in mouse serum and liver obtained by reversed-phase isocratic HPLC. Detection was at 325 nm. Peaks: ROL, retinol; RL, retinyl linoleate; RO, retinyl oleate; RP, retinyl palmitate; RS, retinyl stearate; RH, retinyl hexanoate (internal standard).

Capacity factors and relative retention indices (RRI) are also presented in Table 1. RRI were constant for a given type of column; for a number of heavily used columns of the same brand, using the mobile phase described in this report, capacity factors (and plate count) varied widely from column to column, but RRI

Table 1. Chromatographic characteristics of retinoids, tocopherols, and carotenoids

Compound	Chromatographic characteristics		Limit of detection		Reported ranges for humans[b]	
	Capacity factor	Relative Retention Index[a]	pmol	ng	μmol liter^{-1}	ng ml^{-1}
Retinol	0·70	1664	0·21	0·06	1·6–3·4	456–973
γ-Tocopherol	2·58	2325	3·6	1·50		
α-Tocopherol	3·02	2410	6·4	2·70	16–36	6 800–15 600
Retinyl palmitate	8·59	2966	1·8	0·50		
Lutein	0·96	1858	ND			
Zeaxanthin	1·43	2006	ND			
β-Cryptoxanthin	3·43	2468	ND			
Lycopene	3·99	2563	ND			
α-Carotene	6·61	2835	0·8	0·45		
β-Carotene	7·28	2885	0·8	0·45	0·3–1·1	151–616
Internal standards						
Retinyl hexanoate	1·41	2030				
β-Apo-8'-carotenyl decanoate	4·88	2660	0·6	0·25		

[a] Retention relative to alkylphenone standards; acetophenone = 800.
[b] These ranges are cited in De Leenheer *et al.* (1988).
ND, Not determined.

varied only 4% for retinol, 2% for α-tocopherol and 1% for β-carotene (Furr, H. C. unpublished). Capacity factors for the isocratic system described here ranged from 0·7 for retinol to 7·28 for β-carotene and 8·59 for retinyl palmitate, the least polar analytes expected in these serum analyses.

Serum samples from the National Institute of Standards and Technology (NIST; Gaithersburg, MD) interlaboratory proficiency testing program were analyzed by the techniques reported here. For a series of four NIST 'Round Robin' samples, biases from the group average for retinol were +11·7% (group average 0·23 μg ml^{-1}), +4·0% (0·29 μg ml^{-1}), −12·2% (0·52 μg ml^{-1}), and −9·5% (0·74 μg ml^{-1}); for β-carotene in these samples, bias from the group average was +17·9% (group average 0·19 μg ml^{-1}), −0·6% (0·52 μg ml^{-1}), 0·0% (0·97 μg ml^{-1}), and +1·0% (1·15 μg ml^{-1}). Biases of 0–5% and 5–10% are regarded as excellent and acceptable, respectively, thereby suggesting that the present method of analysis is reliable.

DISCUSSION

The HPLC analysis described in this communication is capable of simultaneously analyzing serum carotenoids, retinoids and tocopherols using one extraction procedure, two detectors and a small amount (10–100 μl) of serum or plasma. In sera of populations in developing countries, many carotenoids such as α-carotene, β-cryptoxanthin, are barely present. For analysis of carotenoids in sera of such populations, a larger volume of serum should be used for extraction. Using a higher attenuation, the carotenoids in an extract from 500 μl of human serum dissolved in 100 μl of final injection solvent could be analyzed without any observed differences. Baseline separation of α-carotene and β-carotene was achieved, and even partial separation of the *cis* isomers of the two carotenes was noted. The identification of the *cis* isomer of carotenes was based on observations by Lesellier *et al.* (1989), Krinsky *et al.* (1990) and Khachik *et al.* (1991), who found that *cis* isomers of α- and β-carotenes eluted after the *trans* isomers. Lycopene, which usually eluted as a broad peak in the authors' previous study (Barua *et al.*, 1989), tended to resolve into several peaks, probably *cis* isomers. It is not clear whether these isomers are naturally present, or are a result of isomerization during the extraction procedure. Standard lycopene, however, showed only a single peak during HPLC, indicating that isomerization probably did not occur during passage through the column. The lycopene peak has been resolved into *cis* and *trans* forms by others (Nelis & De Leenheer, 1983; Cantilena & Nierenberg, 1989). Although precryptoxanthin and β-cryptoxanthin in human serum were partially separated by others (Bieri *et al.*, 1985; Cantilena & Nierenberg, 1989), good resolution (baseline separation) of this carotenoid pair was observed in the present study. Recently, precryptoxanthin in human serum has been characterized as α-cryptoxanthin (Krinsky *et al.*, 1990; Khachik *et al.*, 1991). Furthermore, good separation of the two unidentified carotenoids 'X' and 'Y' (Barua *et al.*, 1989) was also obtained during the present study. It is not known if carotenoid 'Y' the authors found in human serum is the same as 2',3'-anhydrolutein, recently characterized in human serum by Khachik *et al.* (1991). Further work will be necessary to determine if carotenoid 'X' that the authors found in human serum is the same as the *cis* isomer of lutein or zeaxanthin characterized recently in human serum by Krinsky *et al.* (1990).

Although the major vitamin A esters (retinyl palmitate, oleate and stearate) that are found in most biological samples are sufficiently well separated from the major carotenoids to allow simultaneous analysis, significant amounts of carotenoids in sample extracts will also show absorbance at 300 nm. Improper identification of carotenoid absorbances as retinyl ester peaks can be avoided by monitoring absorbances at both 300 and 450 nm, either simultaneously or in sequential runs. In practice, however, few tissues except human or bovine liver and hypervitaminotic human or bovine serum will have appreciable amounts of both carotenoids and retinyl esters.

BHT, when present in the ethanol used to precipitate proteins in serum, occasionally interfered with the retinol peak (Fig. 2), particularly when the detection was done at 300 nm. The use of antioxidants in ethanol to denature plasma proteins has been extensively reviewed (Nierenberg, 1985; Cantilena & Nierenberg, 1989). No beneficial effect was observed when BHT was included as antioxidant during the extraction procedure. It is the authors' opinion that the large amount of tocopherols normally present in serum (16–36 μmol $liter^{-1}$) should serve this function during the extraction and assay procedure.

The use of an internal standard in the serum during extraction of the nutrients improves the accuracy and reproducibility of the assay (De Leenheer *et al.*, 1988). Medium-chain retinyl esters have been used as internal standards in HPLC analysis of milk and liver vitamin A (Ross, 1981, 1986). Retinyl hexanoate and β-apo-8'-carotenyl decanoate, which can be easily prepared in the laboratory, were well resolved from other sample components under the conditions of assay described in this paper. The spectral characteristics of retinyl esters (λ_{max} 325 nm, molar extinction coefficient 52 275 (Ross, 1981*a*)) and of β-apo-8'-carotenyl esters (λ_{max} 426 nm, molar extinction coefficient 122 600 (Ruegg *et al.*, 1959)) are especially appropriate for use as internal standards. When using other mobile phases, other fatty acid esters of these compounds could be substituted to avoid peak overlaps. Retinyl acetate, which is often used as an internal standard for analyses of retinol and tocopherols could not be used because it elutes too closely to retinol in the authors' system. The relative retention indices were 1755 and 1664 for retinyl acetate and retinol, respectively.

The relative retention indices (RRI; retentions relative

to alkylphenones) were found useful for tentative identification of these micronutrients and for characterization of reversed-phase HPLC stationary phases. RRI for α-tocopherol, γ-tocopherol, γ-lycopene, α-carotene, β-carotene and retinyl palmitate were similar with use of this mobile phase on a column with different stationary phase (Partisil ODS-2, Whatman Chemical Separation, Bridewell, NJ; Furr, H. C., unpublished). In gas chromatography, Kovats retention indices (retention relative to alkane standards) have proved useful for tentative identification of compounds. Similar retention indices have been proposed for qualitative identification in reversed-phase HPLC, using ketoalkanes as well as alkylphenones (Baker & Ma, 1979; Baker *et al.*, 1982; Smith, 1982; Bogusz & Anderjan, 1988). The effects of reversed-phase mobile phase composition and stationary phase composition on retention indices of carotenoids and retinoids are currently being studied.

Several reversed-phase HPLC systems have been described for the simultaneous analysis of retinol, tocopherols, and major carotenoids (Miller *et al.*, 1984; Miller & Yang, 1985; Milne & Botnen, 1986; Kaplan *et al.*, 1987; MacCrehan & Schonberger, 1987; Van Haard *et al.*, 1987; Thurnham *et al.*, 1988; Brown *et al.*, 1989). The system described here provides simple (isocratic) operating conditions, has very small sample requirements, requires reasonably short analysis times (less than 30 min chromatography per sample, capacity factor <10 for β-carotene), and provides good resolution of retinol and all major tocopherols and carotenoids in human serum or plasma.

This procedure is currently being adapted for use in large-scale epidemiological studies. Because dichloromethane is volatile, the authors found that its replacement with less-volatile dichloroethane in the final injection solvent and mobile phase did not affect either the position or the resolution of peaks. In both cases, however, a refrigerated autosampler (WISP, Waters) is used, both to reduce sample loss and to minimize the degradation of components. It was also found that if good resolution of carotenoid pairs, like α- and β-carotenes, and α- and β-cryptoxanthins, are not critical, a 15 cm 'Resolve' column can be used for the 30 cm column, and the analysis time can be reduced by half. The HPLC procedure has already been successfully used by several other investigators (Shi *et al.*, 1991; Kirkiles *et al.*, 1992; Lammi-Keefe *et al.* (1992).

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In some recent applications of this method, we have experienced occasional poor recovery of carotenoids which could be completely corrected by addition of 0·1% ammonium acetate to the mobile phase, as suggested by Handelman *et al.*, 1988.

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