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Isocratic rapid liquid chromatographic method for simultaneous determination of carotenoids, retinol, and tocopherols in human serum

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ABSTRACT

An improved isocratic and rapid HPLC method was developed for the measurement of carotenoids, retinol and tocopherols in human serum. Vitamins were extracted with hexane. Mobile phase consisted of a mixture acetonitrile:methylene chloride:methanol with 20 mM ammonium acetate. This method used a small bead size (3 μm) Spherisorb ODS2 column with titane frits. Diode array and fluorescence detectors were used respectively for the detection of carotenoids and retinol/tocopherols. Chromatographic separation was complete in 13 min for β-cryptoxanthin, cis–trans-lycopene, α-carotene, β-carotene, cis-β-carotene, retinol, δ-tocopherol, γ-tocopherol and α-tocopherol. Echinenone and tocol were employed as internal standards for diode array and fluorescence detection. Accuracy was validated using standard reference material (SRM) 968C. Intra-assay and inter-assay precision were respectively 0.2–7.3% and 3.6–12.6%. Sensitivity was verified using the ICH recommendations and the limit of detection (LOD) obtained was sufficient for routine clinical application.

1. Introduction

High-performance liquid chromatographic (HPLC) methods for the separation of fat-soluble vitamins were first reported in 1973 [1]. The separation can be performed using columns with normal phase [2–6] or reverse phase [7–20]. Reverse phase chromatography is a more popular and practical laboratory method to measure tocopherols and carotenoids simultaneously in biological fluids [19,21]. Depending on the analytes of interest, HPLC can be combined with visible, UV (UV or diode-array (DAD)) [9–15,22–26], fluorescence (FLD) [7,8,16,27–32], or electrochemical (ECD) [33–36] detectors or mass spectroscopy (MS) [37,38]. Carotenoids, retinoids and tocopherols can be monitored by UV/DAD detectors, retinoids and tocopherols by FLD, and carotenoids and tocopherols by ECD.

Craft et al. examined the stability and recovery of carotenoids during chromatography and ascertained types of reverse phase columns which proved amenable to simultaneous separation of carotenoids, retinoids and tocopherols [21,27,39–41]. Their survey underscored the importance of trietylamine and ammonium acetate in increasing the yield of carotenoids, particularly α-carotene and β-carotene. They also noted that implementation of biocompatible frits minimizes degradation of the carotenoids during the chromatography. It was also determined that complete chromatographic separation of all relevant species reported in the literature required the use of a gradient, rather than an isocratic solvent. Requirement of a gradient solvent proved disadvantageous given the substantially longer chromatographic times involved relative to the isocratic method. The gradient method necessitates re-balancing of the HPLC system between injections, and various components of the HPLC system (column, pre-column, fitting between pump and column, etc.), can affect the chromatography patterns and significantly modify analyte retention times [21,42].

In the past decade, Craft et al. [16,43–45] have developed and optimized an isocratic chromatography method which utilizes UV–vis and FLD detection to measure retinol, carotenoids and tocopherols. Their method entails use of commercially available chemical "tocol" as internal standard for retinol and tocopherol analyses by fluorescence detection. An oxime form of the β-apo-10′-carotenal which was synthesized in-house served as internal standard for the carotenoids assay under UV detection. Unfortunately, the synthesis protocol of the oxime form of β-apo-10′-carotenal was published with insufficient detail to allow recapitulation of a functional assay in our laboratory. We thus set out to develop a novel, clinical-grade chromatographic method for simultaneous detection of retinoid, carotenoids and tocopherols

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Table 1
Absorption coefficients, wavelengths, and solvents used for preparation of calibrators.

Analyte	Abbr.	Absorption coefficients (dL/g cm)	Wavelength (nm)	Solvent	Reference
Lutein	Lu	2550	445	Ethanol	[20]
Zeaxanthin	Zx	2540	452	Ethanol	[20]
β-Cryptoxanthin	β-Crypt	2356	452	Ethanol	[20]
Echinenone	Echi	2158	458	Hexane	[48]
Lycopene	Lyco	3450	472	Hexane	[20]
α-Carotene	α-Caro	2800	444	Hexane	[20]
β-Carotene	β-Caro	2592	452	Hexane	[20]
Retinol	Vit A	1843	325	Ethanol	[20]
Tocol	Tocol	97	298.5	Ethanol	[49]
δ-Tocopherol	δ-Toc	91	297	Ethanol	[20]
β-Tocopherol	β-Toc	89.4	296	Ethanol	[8]
γ-Tocopherol	γ-Toc	91	298	Ethanol	[20]
α-Tocopherol	α-Toc	75.8	292	Ethanol	[20]
α-Tocopherol acetate	TocAc	40	290	Ethanol	[12]
Retinyl acetate	RetAc	1510	325	Ethanol	[11]

in human serum at our institute's Biomedical Redox Laboratory (S.M.B.D. Jewish General Hospital, Montreal; JGH). Initial attempts to employ echinenone as internal standard for the carotenoids and tocol for the retinol and tocopherol analyses under conditions akin to those reported by Craft et al. [16,43–45] were unsuccessful due to inadequate separation between carotenoids and echinenone. Rapid and efficient separation of principal retinoids, carotenoids and tocopherols in human serum was finally achieved using a novel, isocratic HPLC procedure based on the method of Craft et al. with substitution of oxime form of the β-apo-10′-carotenal, the internal standard and the mobile phase [11,23,24,46,47].

2. Experimental

2.1. Chemicals and reagents

Butylated hydroxytoluene (BHT), ammonium acetate, lycopene, β-carotene, retinyl acetate, retinol, δ-tocopherol, γ-tocopherol, and α-tocopherol acetate were purchased from Sigma (Oakville, Ontario, Canada). Lutein, zeaxanthin, β-cryptoxanthin were purchased from Indofine Chemical Company (Hillsborough, NJ, USA). Echinenone and α-carotene were obtained from Carotenature (Lupsingen, Switzerland). Tocol and α-tocopherol were purchased from Matreya (Pleasant Gap, PA, USA). β-Tocopherol was purchased from Chromadex (Irvine, CA, USA). Acetonitrile, methylene chloride, hexane, methyl alcohol (HPLC grade) and ethyl alcohol (USP grade) were procured from A&C American Chemicals Ltd. (St-Laurent, Quebec, Canada). Standard reference material (SRM) 968C was purchased from National Institute of Standards and Technology (NIST) (Gaithersburg, MD, USA).

2.2. Preparation of standard solutions and calibration

All standards were protected from light and manipulated using amber glass vials. The concentrations of standards were evaluated by spectrophotometry with absorption coefficients as per Table 1 [8,11,12,20,48,49]. Some standard solutions were filtered with Acrodis 25 mm Syringe Filter with 0.1 μm Supor membranes (Life Sciences, Ann Arbor, MI, USA) to remove un-dissolvable components. The purity of each standard was evaluated by HPLC-DAD detection at the same wavelengths used for quantification. For calibrations, standards with purities less than 90% were rejected. The calibration curves consisted of 6 calibrator levels in duplicate injection. Each level contained all standards including two internal standards. To prepare the calibrators series, a stock master calibrator was diluted 1:20; 1:5; 2:5; 3:5; 4:5 with ethyl alcohol containing 30 mg/L BHT and two internal standards. Calibrator solvents were evaporated under a nitrogen-drying manifold at room temperature and reconstituted in the same volume of mobile phase containing 30 mg/L BHT without ammonium acetate before injection. The concentrations of each standard in the master calibrators were 1.27, 0.42, 1.80, 2.70, 0.29, 1.61, 7.60, 0.90, 13.70 and 115 μmol/L for lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene, β-carotene, retinol, δ-tocopherol, γ-tocopherol and α-tocopherol, respectively. Lutein and zeaxanthin were measured together using a mixed preparation calibrator of lutein/zeaxanthin with a ratio 3/1 [23]. Internal standards, echinenone and tocol were 1 and 8 μmol/L in the master calibrator solution.

2.3. Sample treatment and extraction

Blood samples were procured from subjects by venipuncture, centrifuged (1500 × *g*, 10 min, at 4 °C), and serum was separated and aliquoted into amber microcentrifuge tube and stored at −80 °C. In liquid–liquid extraction procedures, samples were thawed at room temperature, gently mixed by inversion and centrifuged at 3000 × *g* at 4 °C for 10 min. Serum aliquots of 200 μL were de-proteinated by addition of 200 μL ethyl alcohol containing 0.5 μmol/L echinenone and 4 μmol/L tocol with 30 mg/L BHT in an amber glass vial. The samples were vortex-mixed for 2 min followed by addition of 800 μL hexane with mixing for 10 min at 1500 rpm by mechanical vortex. The samples were centrifuged for 10 min at 1500 rpm and supernatants were transferred into amber glass vials. The hexane extraction process was repeated. The supernatants were combined and evaporated under nitrogen stream at room temperature. The residues were reconstituted in 100 μL mobile phase containing 30 mg/L BHT without ammonium acetate followed by 2 min vortex and 2 min ultrasonic bath. Twenty microliter samples were injected into HPLC column. The reconstituted residue and injection solvent were stable for a maximum 24 h at 4 °C.

2.4. Chromatographic analysis

HPLC analysis was performed on an Agilent 1100 system (Mississauga, Ontario, Canada) equipped with binary pump G1312A and degasser G1379A, an automatic injector G1367A with cooler G1330B, a heater block G1316A, diode array detector (DAD) G1315B and fluorescence detector (FLD) G1321A. A Waters Rheodyn valve model 7725i with 100 μL loop was used for manual injection. To test the function of ammonium acetate on the recovery of the assay, a Waters Spherisorb ODS2 (4.6 mm × 150 mm, 3 μm) column connected with a 4.6 mm × 20 mm guard column was used. In the final chromatography assays, the column system was a Javelin guard column (3 mm × 20 mm, Spherisorb ODS2 3 μm (C18)) connected to a custom Thermo Spherisorb ODS2 column (3 mm × 250 mm, 3 μm) prepared with titan frits (Life Sciences Canada, Dorval, Quebec,