**Ultra-Performance Liquid Chromatographic Determination of Tocopherols and Retinol in Human Plasma**

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Received 26 November 2012; revised 17 September 2013

A rapid, selective and sensitive ultra-performance liquid chromatography method has been developed for the detection and quantification of tocopherols and retinol in human plasma. Alpha-tocopherol, gamma-tocopherol and retinol are assayed using fluorescence detection. Excitation/emission wavelengths are 295/330 nm and 325/470 nm for the analysis of both tocopherols and retinol, respectively. Retinol acetate is employed as the internal standard. The reversed-phase method incorporates gradient elution with a mobile phase consisting of methanol and acetonitrile. Separation of vitamin compounds is achieved using a bridged ethyl hybrid C18 column. The retention times for retinol, retinol acetate, gamma-tocopherol and alpha-tocopherol are 1.6, 1.8, 3.9 and 4.3 min, respectively. The limits of quantification for retinol, gamma-tocopherol and alpha-tocopherol were 0.02, 0.02 and 0.1 μg/mL, respectively. The assay method is suitable for the analysis of tocopherols and retinol in human plasma. The method may be applied following the ingestion of foods fortified with these fat-soluble vitamins.

**Introduction**

Vitamins commonly found in foodstuffs and nutritional supplements within the human diet are frequently monitored for a variety of applications. Numerous liquid chromatography methods have been published regarding the simultaneous quantification of fat-soluble vitamins in human plasma, including tocopherol and retinol (1–30). Renewed interest and debate has arisen in recent years regarding the possible role of vitamins as antioxidants in the prevention of various disease states, including cardiovascular disease, cancer (31–33) and diabetes mellitus (34). As interest in various vitamin compounds has increased, so too has the need for rapid and reliable methods by which these compounds can be assayed.

Tocopherol and retinol have been assayed in plasma matrices using various stationary phases and methods of detection. Ultraviolet detection of these compounds has been most common in the literature (5–30, 35), but fluorescence detection has also been used in the detection of tocopherol and retinol (23–26). The use of fluorescence detection has allowed for various compounds to be analyzed at low limits of quantification (LOQ) in human plasma as an alternative to ultraviolet detection methods. Fluorescence detection may offer advantages in regard to sensitivity and selectivity (36).

High-performance liquid chromatography methods involving the simultaneous monitoring of tocopherol and retinol in human plasma have often featured chromatography run-times and flow rates requiring considerable consumption of time and/or solvents during sample analyses (2, 7–9, 11, 12, 23, 35). Ultra-performance liquid chromatography (UPLC) allows for enhanced speed and resolution in liquid chromatography. A bridged ethylsilica hybrid column chemistry capable of withstanding up to 15,000 psi of backpressure is used in many Waters UPLC systems. The technology successfully exploits very small particle sizes in column beddings to increase column efficiency and has excellent applicability in the characterization of fat-soluble vitamins within complex matrices beset with endogenous inferences.

Ultra-performance liquid chromatography assays of retinol and/or tocopherol have been developed, but these methods feature ultraviolet detection of these compounds during the course of assay (27–30, 35). We have developed a rapid and sensitive fluorometric UPLC assay for the simultaneous detection and quantification of alpha (α)-tocopherol, gamma (γ)-tocopherol and retinol. The assay has been developed to support pharmacokinetic studies and to support feeding studies requiring the analysis of fat-soluble vitamins.

**Experimental**

**Instrumentation and reagents**

An Acquity Ultra Performance Liquid Chromatograph (Waters Corporation, Milford, MA, USA) was used during assay development. The UPLC was equipped with a quaternary pumping system, a temperature-controlled autosampler unit with a 20-μL loop, photo diode array (PDA) and fluorescence detectors, and Waters Empower 2 software. Alpha- and gamma-tocopherol and all-trans-retinol were purchased from Sigma Aldrich (St. Louis, MO, USA). The internal standard retinol acetate was also purchased from Sigma Aldrich. Alpha-tocopherol, gamma-tocopherol, all-trans-retinol and retinol acetate were ≥96, ≥96, ≥95, and ≥95 purity, respectively. The absorbance maxima of vitamin standards were measured using UPLC PDA-mediated spectrophotometry. The maximum absorbance wavelengths for alpha-tocopherol, gamma-tocopherol and retinol were 295, 292 and 325 nm, respectively. UPLC-grade acetonitrile (99.99%) and methanol (99.9%) were purchased from EMD Chemicals (Philadelphia, PA, USA) through VWR (Suwanee, GA, USA). Hexane (98.5%) and tetrahydrofuran (THF) (99%) used during assay development were analytical grade and were purchased from Mallinckrodt (St. Louis, MO, USA) through VWR.
Methods

Approval for human studies

Collection of human plasma samples described in this manuscript received proper Institutional Review Board approval from the University of Texas MD, Anderson Cancer Center, Houston, TX, USA.

Standard solutions

Molar extinction coefficients for alpha-tocopherol (3270 M$^{-1}$ cm$^{-1}$ at $\lambda = 292$ nm), gamma-tocopherol (3810 M$^{-1}$ cm$^{-1}$ at $\lambda = 298$ nm) (37) and retinol (52,300 M$^{-1}$ cm$^{-1}$ at $\lambda = 325$ nm) (38) were used for determination of quality control concentrations. Standards were injected on the PDA detector for measurement of absorbance units. Molar concentrations of standards were calculated using the Beer-Lambert Law. Stock solutions (2 mg/mL) of retinol, retinol acetate, gamma-tocopherol and alpha-tocopherol were prepared in pure ethanol with 0.04% 3,5-di-tert-butyl-4-hydroxytoluene (BHT) as an additive. BHT was incorporated as an antioxidant to assist in the prevention of vitamin degradation upon atmospheric exposure. Working solutions of each vitamin compound were prepared using pure ethanol with 0.04% BHT additive by serial dilution. All standard solutions were stored at $\sim$80°C before use during assay development. Ethanol was used for the dissolution and storage of all vitamins, as similar to prior investigations (24, 39). All vitamin stock solutions were stored in dark, airtight bottles to inhibit photodegradation (40). In addition, vitamin stores in plasma have been noted to be stable in excess of a year at the storage temperatures and conditions described (18, 41).

Chromatography

Chromatographic separation of vitamin compounds was achieved using an Acquity BEH C18 column (1.7 μm, 2.1 × 150 mm) at 30°C. A Waters Vanguard pre-column (2.1 × 5 mm) preceded the Acquity BEH on the flow path. The autosampler chamber was maintained at a temperature of 10°C during the storage, sampling and injection of processed analytes. The mobile phase used during chromatographic separation consisted of methanol and acetonitrile and featured both gradient elution and a variable flow rate. The methanol/acetonitrile solvent ratio was shifted rapidly from 80/20 (v/v) to 10/90 (v/v) using a linear gradient during the initial 10 s of the chromatographic run using a flow rate of 0.2 mL/min. The flow rate was instantly elevated to 0.4 mL/min, and the methanol/acetonitrile solvent ratio was progressed from 10/90 to 80/20 using a linear gradient that ended at 4 min post-injection. Simultaneous detection of vitamins was observed at fluorescence parameters of 295-nm excitation/330-nm emission wavelengths for both gamma- and alpha-tocopherol, and fluorescence parameters of 325-nm excitation/470-nm emission wavelengths for retinol and retinol acetate. The retention times for retinol, retinol acetate, gamma-tocopherol and alpha-tocopherol were 1.6, 1.8, 3.9 and 4.3 min, respectively, and are depicted in Figures 1 and 2. While the dead time of the assay was $\sim$1 min, the capacity factors of retinol, gamma-tocopherol and alpha-tocopherol were 2.7, 3.1 and 0.48, respectively.

Sample extraction procedure

The extraction procedure used during assay development was adapted and modified from an extraction procedure presented by Siluk and colleagues (24). Twenty microliters of 25 μg/mL retinol acetate internal standard solution was pipetted onto a 100 μL aliquot of human plasma. One hundred microliters of water was added to the sample before the sample was capped and vortex-mixed for 10 s. Three hundred microliters of ethanol containing 0.04% (w/v) BHT was added to the sample vial. The sample vial was capped and vortex-mixed for 10 s. Six hundred microliters of hexane was pipetted into the resulting mixture. The sample vial was capped and vortex-mixed for 3 min before centrifugation at 12,000 rpm for 10 min at 10°C. Using a glass pipet, $\sim$500 μL of hexane supernatant was extracted and collected in a clean sample vial. The collected supernatant was placed into a centrifugal evaporator for $\sim$5 min to evaporate the hexane. Because THF was observed to effectively retrieve dried vitamin residues from glass sample vials during reconstitution, vitamins remaining in the sample vial were reconstituted using 25 μL of THF and 975 μL of ethanol, and the fresh solution was vortex-mixed for 2 min. Ten microliters of reconstituted sample was injected onto the BEH C18 column. All sample preparations occurred in dim light to hinder photolytic degradation processes.

Standard curves and quality controls

Standard curves for retinol and gamma-tocopherol in human plasma were constructed using quality control concentrations of 0.05, 0.2, 0.5, 1, 2, 5 and 10 μg/mL. A standard curve for alpha-tocopherol was also constructed in human plasma using quality control concentrations of 0.2, 0.5, 1, 2, 5 and 10 μg/mL. Tocopherol and retinol endogenous concentrations contained within blank plasma samples were subtracted from corresponding quality control sample concentrations. Vitamin analytes were confirmed through peak purity analyses using PDA detection of analyte spectra. In addition, analytes were identified through chromatographic comparison of analyte unknowns with known pure vitamin compounds, including comparison of retention times. Standard curves for retinol, alpha-tocopherol and gamma-tocopherol were constructed in human plasma by plotting the peak area ratios of each vitamin over the internal standard versus respective vitamin concentrations.

Extraction and recovery

The percent recoveries for retinol, gamma-tocopherol and alpha-tocopherol were determined using the following equation:

$$\text{Concentration of analyte in spiked plasma} \times 100\%$$

$$\text{Concentration of analyte in quality control}$$

$$+ \text{concentration of endogenous analyte in unspiked plasma}$$

Results

Precision and recovery

Spiked plasma concentrations and coefficients of variation are presented in Table I. Spiked plasma concentrations and inter-day coefficients of variation are presented for all quality control
plasma concentrations, including concentrations of 0.05, 0.2, 0.5, 1, 2, 5 and 10 μg/mL for retinol and gamma-tocopherol and including concentrations of 0.2, 0.5, 1, 2, 5 and 10 μg/mL for alpha-tocopherol. In addition, intra-day coefficients of variation are reported in Table I at concentration levels of 0.05, 0.5 and 5 μg/mL for gamma-tocopherol and retinol and at concentration levels of 0.5 and 5 μg/mL for alpha-tocopherol. Percent recovery data are presented in Table II. The data are reported at concentration levels of 0.05, 0.5 and 5 μg/mL for gamma-tocopherol and retinol and at concentration levels of 0.5 and 5 μg/mL for alpha-tocopherol. Spiked plasma concentrations were measured to be within 87.8 and 99.8%, within 92.9 and 99.8% and within 91.9 and 97.6% of ethanol-dissolved quality controls of all-trans-retinol, gamma-tocopherol and alpha-tocopherol, respectively.

**LOQ and LOD**

The limit of quantification for all compounds was determined from plasma standards as the lowest concentration for which the analyte signal-to-noise ratio was > 10. Similarly, the limit of detection for all compounds was determined as the lowest

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**Table I**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration level (μg/mL)</th>
<th>Spiked concentration found (μg/mL)</th>
<th>% Found (n = 5)</th>
<th>Inter-day % (n = 5)</th>
<th>Intra-day % (n = 3)</th>
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<tr>
<td>Retinol</td>
<td>0.05</td>
<td>0.0561</td>
<td>87.8</td>
<td>4.40</td>
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<td></td>
<td>0.2</td>
<td>0.207</td>
<td>96.4</td>
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<td></td>
<td>0.5</td>
<td>0.501</td>
<td>99.8</td>
<td>2.46</td>
<td>2.93</td>
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<td></td>
<td>1</td>
<td>0.933</td>
<td>93.3</td>
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<td></td>
<td>2</td>
<td>1.98</td>
<td>99.1</td>
<td>11.6</td>
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<td>5.14</td>
<td>97.2</td>
<td>5.89</td>
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<td></td>
<td>10</td>
<td>9.76</td>
<td>97.6</td>
<td>12.0</td>
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<tr>
<td>γ-Toc.</td>
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<td>0.0464</td>
<td>92.9</td>
<td>5.77</td>
<td>9.19</td>
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<td></td>
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<tr>
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<td>0.0465</td>
<td>93.1</td>
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<td>5</td>
<td>5.24</td>
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<td>6.58</td>
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<td>10</td>
<td>9.98</td>
<td>99.8</td>
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<tr>
<td>α-Toc.</td>
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<td>0.212</td>
<td>94.0</td>
<td>9.00</td>
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<td>0.5</td>
<td>0.513</td>
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<td>97.6</td>
<td>4.31</td>
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</table>
concentration for which the analyte signal-to-noise ratio was $>3$. In the case of retinol, gamma-tocopherol and alphatocopherol, the LOQ were 0.02, 0.02 and 0.1 $\mu$g/mL, respectively. The limits of detection (LOD) for the aforementioned vitamins were 0.01, 0.01 and 0.05 $\mu$g/mL, respectively.

**Linearity**

Standard curves for retinol, gamma-tocopherol and alphatocopherol yielded correlation coefficients of $r^2 = 0.9992$, $r^2 = 0.9988$ and $r^2 = 0.9978$, respectively, and are presented in Table III.

**Discussion**

**Method analysis**

Among UPLC investigations that characterize tocopherol and/or retinol in human plasma (27–30), this method features gradient elution of all analytes in plasma in similar fashion to a handful of methods (28, 30). However, in contrast to the cited methods, this method uses fluorescence detection for the characterization of tocopherols and retinol in human plasma. The cited investigations feature the use of UPLC separation technology in tandem with ultraviolet detection.

Inter-day and intra-day coefficients of variations and percent recoveries listed in Tables I and II are similar to data reported from various investigations involving analyses of retinol, gammatocopherol and alpha-tocopherol using HPLC with fluorescence detection (23–26). With reference to plasma concentrations for retinol, the cited investigations reveal inter-day coefficient of variations ranging from 3.9 to 5.6%, intra-day coefficient of variations ranging from 2.2 to 7.9% and percent recoveries ranging from 95 to 101%. With reference to alpha-tocopherol, the aforementioned investigations report inter-day coefficient of variations between 3.2 and 8.2%, intra-day coefficient of variations between 2.4 and 13.4% and percent recoveries between 97 and 106%. Furthermore, gamma-tocopherol plasma concentration data following HPLC separation with fluorescence detection reveal inter-day coefficients of variation, intra-day coefficients of variation and percent recoveries ranging from 3.8 to 6.2%, 2.8 to 10.3% and 101 to 109%, respectively (23–26).

This method’s LOQ and detection are lower than those of several HPLC/fluorescence (24–26) and UPLC/UV citations (27–30). Limits of quantification using HPLC methods range broadly from 10 to 30 ng/mL, from 4 to 500 ng/mL and from 2 to 250 ng/mL, respectively, for retinol, alpha-tocopherol and gamma-tocopherol. Limits of detection are reported as low as 1, 20 and 3 ng/mL for retinol, alpha-tocopherol, and gamma-tocopherol, respectively (24). Investigations featuring UPLC/UV methods report comparatively higher LOQ and LOD data than surveyed HPLC/fluorescence assays, with LOQs as low as 46 ng/mL (for retinol) and LOD as low as 18 ng/mL (for gamma-tocopherol). It should be noted that the quantification limits described by our method are less than (25–30) or equal to (24) the limits reported by all but one investigation (23). However, the quantification limit described for this method will easily accommodate the lower ends of human plasma concentration normal ranges of all vitamins described.

Despite the aforementioned similarities of this method to that of previous investigations describing the analysis of these vitamins, this method possesses multiple features that support its application. For instance, this method requires a relatively small plasma sample volume, in contrast to several investigations that employ larger plasma aliquots during vitamin extraction steps (27–30). In addition, this method employs a smaller total extraction volume than those employed by several methods, some of which greatly exceeding 1 milliliter in volume (24, 27–29). This method also features a considerably lower flow rate than several methods (23, 24, 26, 28, 29) and features a considerably shorter run time than multiple methods (23, 24, 26), thus promoting conservative solvent consumption and rapid sample analysis.

**Internal standard**

The liquid–liquid extraction internal standard, retinol acetate, was deemed a suitable internal standard for this assay because of its documented use as suitable internal standard in previous investigations involving fat-soluble vitamins, including both retinol and the tocopherols (13–15, 17, 24).

**Gradient elution**

This assay features a variation of solvent ratio and flow rate to achieve optimal separation of all vitamins using multiple detection channels. A linear gradient was employed during analysis. The gradient shifted the methanol/acetonitrile ratio from 80/20 (v/v) to 10/90 (v/v), followed by a gradual reversion to initial conditions. The linear gradient employed during the initial chromatographic run phase allowed acceptable resolution of gamma- and alpha-tocopherol and allowed acceptable resolution of retinol and retinol acetate on their respective channels. Although the gradient transition featured gradually weakening mobile phase strength, marginal analyte peak broadening was observed in face of considerable peak separation benefits. Alternative mobile phase ratios and gradients employed during
method developed resulted in merged analyte peaks, particularly in the case of alpha- and gamma-tocopherols. The methanol/ acetonitrile solvent ratio and flow rate (0.2 to 0.4 mL/min) shifts featured during the gradient were also useful in creating separation of analyte peaks from the solvent front and endogenous compound interferences in plasma.

**Method application toward clinical samples**

The vitamin assay method was applied toward the "Women into the African-American Nutrition for Life" (A NULIFE) Study (42), a dietary and behavioral intervention investigation conducted at the University of Texas, MD, Anderson Cancer Center in the Center for Research on Minority Health. A NULIFE examined the impact of the dietary intake of high-fiber and low-fat foods on breast cancer prevention. Among other intervention factors, the vitamin intakes of volunteers were assessed. One hundred and sixty-four volunteers took part in the study. Volunteers provided plasma containing basal vitamin concentrations and vitamin concentrations associated with vitamin supplementation. Plasma samples assayed revealed tocopherol and retinol concentrations within normal ranges for humans, and all analyte concentrations exceeded the lower LOQ described in this assay. Mean retinol, alpha-tocopherol and gamma-tocopherol concentrations in \( n = 363 \) plasma samples were \( 0.524 \pm 0.193, 5.32 \pm 1.68 \) and \( 1.23 \pm 0.490 \mu g/mL \). The plasma concentrations observed for these compounds were consistent with reported plasma concentrations in healthy human adults (43). A representative sample of study data is included in Table IV.

**Conclusion**

The fat-soluble vitamins assessed during this investigation are commonly consumed in the human diet, either via a multiplicity of food sources or via vitamin supplementation. The widespread monitoring of dietary intakes and related applications has resulted in the need for fast and reliable assay techniques for vitamin assessment. The UPLC method described in this manuscript was used in the analysis of patient plasma samples in a recent vitamin intervention study. The method proved rapid, reproducible, sensitive and selective for the simultaneous determination of gamma- and alpha-tocopherol upon which the study was focused. The method is applicable to human clinical studies requiring the assay of these vitamins in plasma.

**Acknowledgments**

We thank Dr. Lovell A. Jones, PhD and Dr. Denae W. King, PhD at the University of Texas, MD, Anderson Cancer Center, Center for Research on Minority Health, for their contribution and guidance regarding A NULIFE project proceedings.

**Funding**

This work was performed using research infrastructure associated with the Research Centers in Minority Institutions (RCMI) program at Texas Southern University. Research infrastructure support [grant numbers G12RR003045-21 and CO6 RR012537] from the National Institute on Minority Health and Health Disparities (NIMHD), National Institutes of Health (NIH), Department of Health and Human Services (DHHS) and its contents are solely the responsibility of the authors and do not necessarily represent the official views of NIMHD or NIH.

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