



Fluorometric Determination of Vitamin Constituents in Human Plasma Using Ultra Performance Liquid Chromatography

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Abstract

Purpose: A rapid, selective and sensitive ultra performance liquid chromatography method has been developed for the detection and quantification of several vitamins in human plasma.

Methods: Alpha tocopherol, gamma tocopherol, and retinol are assayed using fluorescence detection. Excitation/emission wavelengths are 295 nm/330 nm and 325 nm/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.

Results: The retention times for retinol, retinol acetate, gamma tocopherol and alpha tocopherol are 1.6 minutes, 1.8 minutes, 3.9 minutes and 4.3 minutes, respectively. The limits of quantification for retinol, gamma tocopherol and alpha tocopherol, were 0.02 µg/ml, 0.02 µg/ml, and 0.1 µg/ml, respectively.

Conclusion: The assay method is suitable for the analysis of these vitamins in human plasma following the ingestion of foods fortified with these fat soluble vitamins.

Keywords: Fluorescence; Retinol; Tocopherol; Ultra performance; UPLC

Introduction

Vitamins commonly found in foodstuffs and nutritional supplements within the human diet are frequently monitored for a variety of applications. Numerous high performance liquid chromatography methods have been published regarding the simultaneous quantification of fat soluble vitamins, including tocopherol and retinol. 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 [1-3], and diabetes mellitus [4]. 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, including mass spectrometry [5-8]. Although normal phase methods have been employed for the separation of some compounds [9-13], the vast majority of high performance liquid chromatography based fat soluble vitamin assay methods have employed reversed phase chromatography to facilitate analyte separation [14-47]. Ultraviolet detection of these compounds has been most common in the literature [9-39,42-47], but fluorescence detection has also been used in the detection of tocopherol and retinol [32-42]. The use of fluorescence detection has allowed for various compounds to be analyzed at low limits of quantification and detection in plasma-human [32-37,40] or otherwise [39] as an alternative to ultraviolet detection methods. Fluorescence detection offers considerable advantages of superior sensitivity and selectivity, due to its trademark relative low background noise and ability to single out organic molecules that are detectable by multiple wavelengths. An additional advantage of fluorescence detection methods is its ability to achieve appreciable quantitative analysis even in the instance of less than optimal column resolution [48].

Several methods have been developed for the simultaneous monitoring of tocopherol and retinol in human plasma [5-8,13-

27,29,30,32-37,40,43-46]. These methods have often featured chromatography run-times and flow rates requiring considerable consumption of time and/or solvents during sample analyses [16-18, 20,21,29,35,47]. Ultra performance liquid chromatography (UPLC) allows for enhanced speed and resolution in liquid chromatography. A bridged ethylsiloxane-silica hybrid (BEH) 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 interferences.

There are several literature citations that have described the development of ultra performance liquid chromatography assays for retinol and/or tocopherol [41-47]. We have developed a rapid and sensitive fluorometric assay for the simultaneous detection and quantification of alpha (α)-tocopherol, gamma (γ)-tocopherol, and retinol using ultra performance liquid chromatography. The assay has been developed to support pharmacokinetic studies and feeding studies requiring the analysis of fat soluble vitamins.

Experimental

Chemicals

Alpha and gamma tocopherol, and all-trans-retinol were purchased

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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 $\geq 96\%$, $\geq 96\%$, $\geq 95\%$, and $\geq 95\%$ purity, 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 (99%) used during assay development were analytical grade, and were purchased from Malinckrodt (St. Louis, MO, USA) through VWR (Suwanee, GA, USA).

Human studies approval

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

Standard solutions

2 mg/mL stock solutions of retinol, retinol acetate, gamma tocopherol, and alpha tocopherol were all 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 -80°C before use during assay development. Ethanol was observed to be a suitably soluble and stable solvent for the dissolution and storage of all vitamins, as noted during prior investigations [36,49]. All vitamin stock solutions were stored in dark, airtight bottles to inhibit photodegradation [50]. In addition, vitamin stores in plasma have been noted to be stable in excess of a year at the storage temperatures and conditions described [51,52].

Chromatography

An Acquity Ultra Performance Liquid Chromatography (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 and fluorescence detectors, and Waters Empower 2 software. Chromatographic separation of vitamin compounds was achieved using an Acquity BEH C18 column (1.7 μm , 2.1 x 150 mm) at 30°C Celsius (C). A Waters Vanguard pre-column (2.1 x 5 mm) was preceded 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 seconds 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 minutes 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 minutes, 1.8 minutes, 3.9 minutes and 4.3 minutes, respectively, and are depicted in Figure 1 and Figure 2. While the dead time of the assay was approximately 1 minute, 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 [36]. Twenty microliters of 25 $\mu\text{g}/\text{mL}$ retinol acetate internal standard solutions 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 seconds. 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 seconds. Six hundred microliters of hexane was pipetted into the resulting mixture. The sample vial was capped and vortex mixed 3 minutes before centrifugation at 12,000 rpm for 10 minutes at 10°C . Using a glass pipet, approximately 500 μL of hexane supernatant was extracted and collected into a clean sample vial. The collected supernatant was placed into a centrifugal evaporator for approximately 5 minutes to evaporate the hexane. Because tetrahydrofuran (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 tetrahydrofuran and 975 μL of ethanol, and the fresh solution was vortex mixed for 2 minutes. 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 were constructed in human plasma using quality control concentrations of 0.05, 0.2, 0.5, 1, 2, 5, and 10 $\mu\text{g}/\text{mL}$. A standard curve for alpha tocopherol was also constructed in human plasma using quality control concentrations

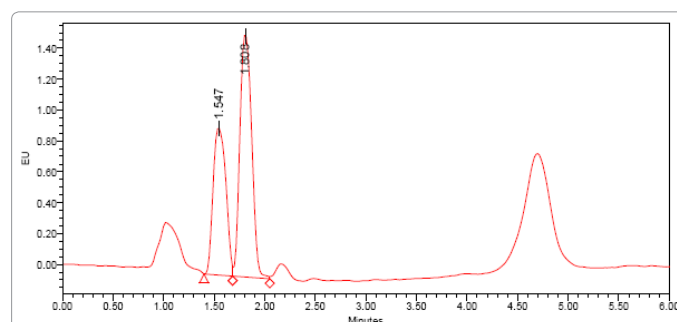


Figure 1: A representative plasma chromatogram depicting 0.5 $\mu\text{g}/\text{mL}$ concentrations of retinol and retinol acetate at 1.5 minutes at 1.8 minutes, respectively.

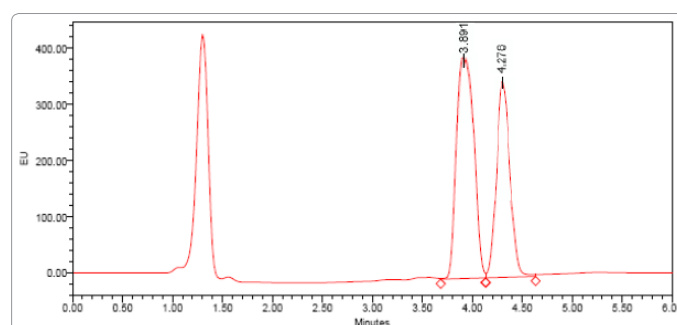


Figure 2: A representative plasma chromatogram depicting 5 $\mu\text{g}/\text{mL}$ concentrations of gamma and alpha tocopherol at 3.9 and 4.3 minutes, respectively.

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 photo diode detection of analyte spectra. In addition, analytes were identified through their comparison with known pure vitamin compounds with respect to analyte retention time.

Results

Extraction and recovery

The post-extraction recoveries for retinol, gamma tocopherol and alpha tocopherol were determined through (n=5) independent observations using concentration levels of 0.05, 0.5 and 5 µg/mL for gamma tocopherol and retinol, and using concentrations of 0.5 and 5 µg/mL for alpha tocopherol. In addition, intra-day extraction variability values were calculated using (n=3) quantitations of analyte plasma concentration levels recorded during a single day, whereas inter-day extraction variability values were calculated using (n=5) quantitations of concentration levels recorded during several days. In similar fashion to post-extraction recovery calculations, concentration levels of 0.05, 0.5 and 5 µg/mL for gamma tocopherol and retinol, and concentration levels of 0.5 and 5 µg/mL for alpha tocopherol were used for intra-day and inter-day calculations (Table 1). The post-extraction recoveries for retinol, gamma tocopherol and alpha tocopherol were determined by accounting for their presence in unspiked human plasma. Percent recovery was calculated by multiplying 100 times vitamin concentration in spiked plasma, dividing its product by the sum of vitamin concentration in pure quality controls and vitamin concentration in unspiked plasma.

Linearity

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. Standard curves for retinol, gamma tocopherol and alpha tocopherol yielded correlation coefficients of $r^2=0.9992$, $r^2=0.9988$ and $r^2=0.9978$, respectively. (Refer to Table 2 for standard curve information).

Accuracy and precision

The accuracy of the assay ranged from 87.8% to 99.8% (n=5), from 92.9% to 99.8% (n=5), and from 91.9% to 97.6% (n=5) for all-trans-retinol, gamma tocopherol, and alpha tocopherol, respectively. Intra-day and inter-day coefficients of variation for low, medium, and high concentration samples of the vitamins are presented in Table 1.

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 greater than ten. Similarly, the limit of detection

for all compounds was determined as the lowest concentration for which the analyte signal to noise ratio was greater than three. In the case of retinol, gamma tocopherol and alpha tocopherol, the limits of quantification were 0.02 µg/ml, 0.02 µg/ml, and 0.1 µg/ml, respectively. The limits of detection for the aforementioned vitamins were 0.01 µg/ml, 0.01 µg/ml, and 0.05 µg/ml, respectively.

Discussion

UPLC method analysis

The ultra performance liquid chromatography method describing the analysis of tocopherols and retinol yielded comparable yet distinctive results from several analogous ultra performance methods [41-47]. This method focused on the quantitation of these analytes in a human plasma matrix, in contrast to investigations utilizing UPLC technology for the characterization of these analytes in other animal species [47], and in matrices aside from human plasma [41,42]. Among UPLC investigations that characterize tocopherol and/or retinol in human plasma [43-46], this method features gradient elution of all analytes in plasma in similar fashion to a handful of methods [44,46]. However, this ultra performance liquid chromatography method is unique from previous UPLC-driven analyses of vitamin constituents in its use of fluorescence detection for the characterization of tocopherols and retinol in a human plasma matrix. In previous human studies involving these compounds, the use of fluorescence detection in tandem with UPLC separation technology had not been reported as a means of analyte characterization. Use of fluorescence detection provides the advantage of superior selectivity of these analytes as compared to ultraviolet detection in plasma, in addition to the advantages of short chromatographic run time and superior resolution often associated with UPLC analysis. Furthermore, this UPLC method yielded comparable to superior analyte detection sensitivity in comparison to previous HPLC assays in human plasma using fluorescence detection [32-37,40].

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 [22-24,26,36].

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 80/20 (v/v) methanol/acetonitrile ratio was observed as best suited for initial incorporation of drug sample into the mobile phase. 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 involving the tocopherol stereoisomers. The methanol/acetonitrile solvent ratio and flow rate (0.2 mL/min to 0.4 mL/min) shifts featured during the gradient were also useful in creating

| Compound | Concentration range | Equation | R ² |
|----------|---------------------|--------------------|----------------|
| Retinol | 0.05- 10 | Y= 1.05x - 0.00210 | 0.9992 |
| G. Toc. | 0.05 -10 | Y= 53.8x - 0.571 | 0.9988 |
| A. Toc. | 0.2 -10 | Y= 16.3x - 1.05 | 0.9978 |

Table 1: Standard curve equations for of retinol, gamma tocopherol (G. Toc.) and alpha tocopherol (A. Toc.) in human plasma.

| Compound | Concentration (µg/mL) | Accuracy | | Precision | | Recovery (n=5) % | |
|----------|-----------------------|---|------------------|-------------------|-------------------|------------------|------|
| | | Concentration found (mean ± SD) (µg/mL) | % Accuracy (n=5) | Inter-day % (n=5) | Intra-day % (n=3) | | |
| Retinol | 0.05 | 0.0561 | 87.8 | 4.40 | 3.18 | 98.0 | |
| | 0.2 | 0.207 | 96.4 | 3.05 | | | |
| | 0.5 | 0.501 | 99.8 | 2.46 | | | |
| | 1 | 0.933 | 93.3 | 4.14 | 2.93 | 93.6 | |
| | | 2 | 1.98 | 99.1 | | | 11.6 |
| | | 5 | 5.14 | 97.2 | | | 5.89 |
| | | 10 | 9.76 | 97.6 | | | 12.0 |
| G. Toc. | 0.05 | 0.0464 | 92.9 | 5.77 | 9.19 | 107 | |
| | 0.2 | 0.205 | 97.5 | 8.99 | | | |
| | 0.5 | 0.0465 | 93.1 | 6.76 | | | |
| | 1 | 1.02 | 98.4 | 2.01 | 5.42 | 101 | |
| | | 2 | 2.03 | 98.5 | | | 13.2 |
| | | 5 | 5.24 | 95.2 | | | 6.58 |
| | | 10 | 9.98 | 99.8 | | | 11.5 |
| A. Toc. | 0.2 | 0.212 | 94.0 | 9.00 | 6.41 | 101 | |
| | 0.5 | 0.513 | 97.5 | 13.1 | | | |
| | 1 | 0.919 | 91.9 | 5.46 | | | |
| | 2 | 1.86 | 92.8 | 9.58 | 4.72 | 96.1 | |
| | | 5 | 5.19 | 96.2 | | | 9.63 |
| | | 10 | 9.76 | 97.6 | | | 4.31 |

Table 2: Intra-day and inter-day precision and accuracy of retinol, gamma tocopherol (G. Toc.), and alpha tocopherol (A. Toc.) in human plasma.

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 [53], a dietary and behavioral intervention investigation conducted at the University of Texas M.D. 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 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 limits of quantitation described in this assay.

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. Although many high performance liquid chromatography methods have been employed in the analysis of such compounds, ultra performance liquid chromatography has been employed relatively sparingly in the interest of vitamin assay. 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.

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