Determination of Retinol and Tocopherols in Human Serum using Ultra-Performance Liquid Chromatography with Photodiode Array

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ABSTRACT

Method that can simultaneously determine retinol, γ-tocopherol and α-tocopherol in human serum was developed utilizing ultra-performance liquid chromatography. Retinyl and tocopheryl acetates were employed as internal standards. The reverse-phased method utilizes isocratic elution with a mobile phase consisting of 20% acetonitrile and 80% methanol at a flow rate of 0.800 mL/minute. Separation was attained using an ethylene bridged hybrid (BEH) C18 column. Retinols and tocopherols were detected by photodiode array at wavelengths 325 nm and 295 nm, respectively. The retention times for retinol and retinyl acetate were 0.42 and 0.49 minutes respectively. γ-Tocopherol, α-tocopherol and tocopheryl acetate eluted at 0.86, 0.94 and 1.1 min, respectively. The limits of quantification were determined and found to be 0.025 μg/mL, 0.50 μg/mL and 1.0 μg/mL for retinol, γ-tocopherol and α-tocopherol, respectively. The method has been found to be suitable for the determination of retinol and tocopherols in human serum.

Keywords: Retinol, Tocopherols, UPLC, Photodiode array.

INTRODUCTION

Fat-soluble vitamins including Vitamin E and Vitamin A exhibit high biological activity against oxidants and free radicals[1]. They are commonly found in fats and fatty food components of vegetables and animals. Vitamin E constitutes mixtures of compounds including tocopherol isomers (α, β, γ and δ) and tocotrienols[2]. Among these, the most active is the α-tocopherol. Vitamin E prevents oxidative damage to the cellular structures through breaking reactions of free radicals[3]. Retinol, which is the active form of Vitamin A is necessary for normal vision, growth and development, and maintenance of immune system[4]. Vitamin A deficiency (VAD) may result to night blindness or nyctalopia and remains very high throughout the world[5]. According to the 8th National Nutrition Survey of the Philippines' Food and Nutrition Research Institute, VAD remains a persistent public health issue. An increase of VAD incidence among children, from 15.2% in 2008 to 20.4% in 2013 was noted[6].

Various methods for the analysis of

fat-soluble vitamins in human serum or plasma are available including high-performance liquid chromatography (HPLC) using UV-Visible[7,8,9], fluorescence[10,11,12] and mass spectrometer as detectors[13,14,15]. These methods, however, exhibit some disadvantages and/or limitations[16]. The objective of this study was to develop a rapid, accurate and inexpensive method using ultra-performance liquid chromatography (UPLC) with photodiode array detector for the determination of fat–soluble vitamins, such as Vitamins A and E in human serum which can be used in the nutritional assessment of different population groups.

EXPERIMENTAL

Reagents

Stock solutions of retinol (107.1 ± 5.4 µg/mL) and its internal standard-retinyl acetate (123.7 ± 6.2 µg/mL) in ethanol; and γ-tocopherol (1000 ± 5 µg/mL), α-tocopherol (1000 ± 5 µg/mL) and their internal standard-tocopheryl acetate (1000 ± 5 µg/mL) in methanol were purchased as 1 mL ampoule from Cerilliant (Texas, USA). HPLC grade methanol, acetonitrile, ethanol and hexane were obtained from Merck (Darmstadt, Germany). Analytical grade butylated hydroxytolune (BHT) and tetrahydrofuran (THF) were obtained from Sigma-Aldrich (Missouri, USA). Two levels of certified reference materials (CRMs)-Vitamin A Plus E Low and High (pooled normal human serum) were purchased from UTAK Laboratories, Inc. (California, USA).

Instrumentation

Chromatographic analysis was done using a Waters Acquity Ultra-Performance Liquid Chromatography H-Class System (Massachusetts, USA). The UPLC was assembled with a quaternary solvent manager capable of handling up to 15,000 psi, temperature-controlled sample manager (flow through needle) and photo diode array eλ detector. The photodiode array is capable of simultaneous detection at different wavelengths in one run. Data were collected and analyzed using Waters Empower 3 software.

Spectrophotometric analyses were performed on Agilent Technologies Cary- 60 UV-Vis Spectrophotometer (California, USA). Data were collected and analyzed using Cary WinUV software.

Standards Preparation

Working solutions of retinol and tocopherols, including their internal standards were prepared in ethanol with 0.04% (w/v) butylated hydroxytoluene (BHT) daily. BHT acts as antioxidant to prevent vitamin degradation upon atmospheric exposure. Concentrations of retinol and retinyl acetate were verified spectrophotometrically using molar absorptions of 52480 $M^{-1}cm^{-1}$ and 52680 $M^{-1}cm^{-1}$ at 325 nm for retinol and retinyl acetate, respectively. Standard curve for retinol was constructed using the calibration solutions with concentrations 0.05, 0.10, 0.25, 0.50, 1.0, 2.0 and 3.0 µg/mL. On the other hand, calibration solutions containing 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 µg/mL γ-tocopherol and 1.5, 3.0, 5.0, 10, 20, 30 and 40 µg/mL α- tocopherol were also constructed. All standard solutions were stored in amber glass vials and at -20ºC prior to analysis.

Vitamin Extraction

All procedures were performed in dim light to avoid vitamin degradation. In a 13x100 mm screw- capped test tube, 500 µL of the standard or CRMs were vortex- mixed with 30 µL of 12 µg/mL retinyl acetate (IS) and 30 µL of stock tocopheryl acetate (IS) for 30 seconds. Five hundred microliters of cold ethanol with 0.04% (w/v) BHT was pipetted to the test tube. It was screw- capped and mixed vigorously using a vortex mixer for 3 minutes. In the resulting mixture, 2500 µL of cold hexane was added. The test tube was screw- capped and then mixed vigorously for another 3 min prior to centrifugation at 4000 rpm for 10 min at 0ºC. Using a pipette, around 2000 µL of upper-hexane layer was collected and transferred into a clean 4 mL-amber glass vial. Hexane was evaporated to dryness under nitrogen gas at 60ºC. Vitamins were resuspended using 10 µL tetrahydrofuran (THF) and 290 µL ethanol. The reconstituted sample was vortex-mixed for 2 minutes. Two microliters of the reconstituted sample was injected onto the separation column.

Chromatographic Analysis

Separation of vitamins was accomplished using a 2.1 x 100 mm Acquity ethylene bridged hybrid (BEH) C18 column (1.7 µm particle size) at 30ºC. The autosampler compartment was maintained at 15ºC. Methanol and acetonitrile (80:20, v/v) were used as mobile phase at a constant flow rate of 0.800 mL/minute. Maximum absorbance of vitamins was observed at a wavelength of 325 nm and 295

nm, for retinols and tocopherols, respectively. The total analysis was set for 3 minutes. Concentration of individual vitamins was determined from the plot of peak area ratios of each vitamin and the corresponding internal standard against the vitamin concentrations.

RESULTS AND DISCUSSION

Vitamins were extracted from serum using liquid- liquid extraction (LLE). Cold ethanol was used as protein-precipitating agent while hexane was used as extracting solvent. Based from previous study which compared different organic solvents to extract the fat-soluble vitamins in humans serum, acceptable recoveries were obtained from using hexane. However, two-step extraction which involves hexane and dichloromethane could improve the percent recoveries[9].

Different chromatographic parameters such as separation column, column temperature, mobile phase composition, flow rate, and wavelength were optimized to obtain peaks with a best shape and resolution while keeping the retention time at minimum. The most desirable results were obtained using the conditions described above. Fat-soluble vitamins were separated using an isocratic elution involving methanol and acetonitrile as solvents. Fig. 1 shows the representative chromatogram of different vitamins analyzed. The retention times for retinol and retinyl acetate were 0.42 and 0.49 min respectively. On the other hand, the retention times for γ-tocopherol, α-tocopherol and tocopheryl acetate, were 0.86, 0.94 and 1.1 min respectively. Retinyl palmitate was also tested as internal standard and eluted at around 2.2 minutes. However, it was detected in the serum sample; hence, failed the criteria for being an internal standard. Therefore, run time can be reduced to two minutes. The retention times found were lower than previous UPLC investigations, hence, exhibit faster run time[10,15,17].

Standard curves for retinol, γ-tocopherol and α-tocopherol were linear over the tested concentration range and generated correlation coefficients of $r^2 = 0.9993$, $r^2 = 0.9974$ and $r^2 =$ 0.9982, respectively. Regression equations obtained as a mean of seven calibration curves are presented in Table 1. The concentration ranges encompass the reference values[18,19] of retinol (0.113 to >1.20 µg/mL), γ-tocopherol (0 to 6 µg/mL) and α-tocopherol (3.8 to 17 µg/mL) in human serum and the expected concentrations in certified reference materials. This ensures that concentration of vitamins in human serum can be analyzed at a high degree of accuracy without diluting the sample.

To determine the intra-day precision, under the same conditions described above, certified reference materials (CRMs) were analyzed four times within the day. Similarly, to obtain the inter-day precision, the same CRMs were analyzed on six different days. The percent recoveries and precision are given in Table 2. Presented data are at low and high concentration levels of CRMs. The coefficients of variation were below 7% for intra-day while below 4% for inter-day. The recoveries both for intra-day and inter-day are above 80%. Low values of SD and CV, which are within acceptable range suggest that the current method is accurate and precise. Intra-day and inter-day coefficients of variation are comparable to the data reported from various studies involving analysis of fat-soluble vitamins in human serum[2,15,17].

The limits of quantification (LOQ) defined as the minimum concentration for which the analyte signal-to-noise (S/N) ratio is greater than 10 and could be quantified with sufficient precision and accuracy were determined. Similarly, limits of detection (LOD) defined as the minimum concentration for which the analyte signal-to-noise (S/N) ratio is greater than 3 were determined. The LOQ values for retinol, γ-tocopherol and α-tocopherol were 0.025, 0.50 and 1.0 µg/mL, respectively. Furthermore, the LOD values for retinol, γ-tocopherol and α-tocopherol were 0.01, 0.25 and 0.50 µg/mL, respectively. Both LOQ and LOD were experimentally verified by seven replicate injections of the vitamin standard concentrations. Accuracy and precision were within acceptable range. The LOQ and LOD values determined from this method were deemed fit for purpose. However, these values can still be improved by optimizing the parameters such as extraction procedures, chromatographic variables and lowering the calibration ranges.
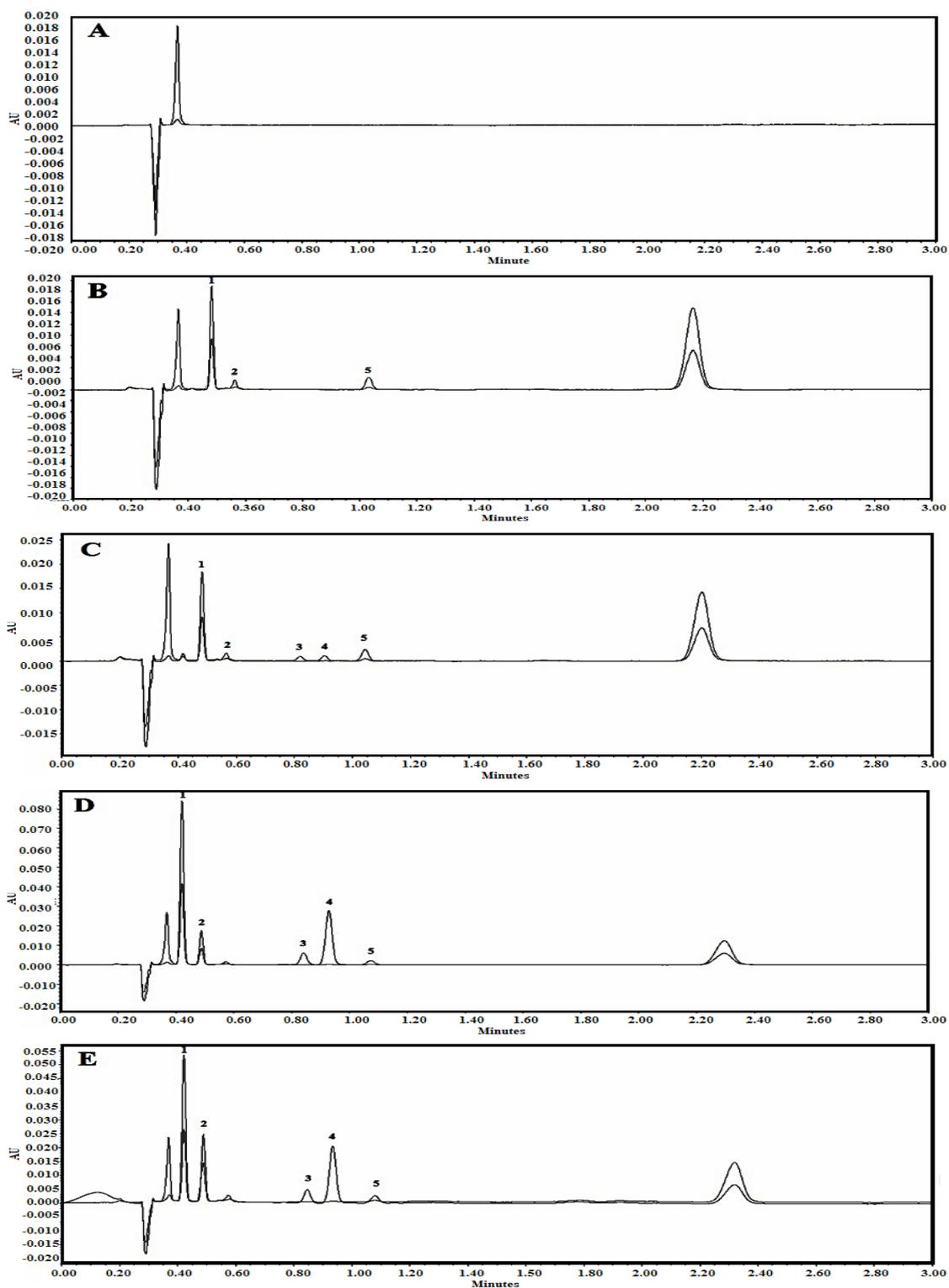
Fig. 1. Representative overlay of chromatograms at different wavelengths (blue at 325 nm and black at 295 nm) of solvent blank (A), blank with internal standards (B), lowest calibration standard solution (C), highest calibration standard solution (D) and human serum sample (E) . Peaks: retinol (1), retinyl acetate (2), γ-tocopherol (3), α- tocopherol (4) and tocopheryl acetate (5)

Table 1: Calibration curve equations for the vitamins analyzed (n=7)

Vitamin	Concentration range (µg/mL)	Linear Equation	Mean slope (95% confidence interval)	Mean intercept (95% confidence interval)	Correlation coefficient, r^2
Retinol	0.05 - 3.0	y = 1.5028x + 0.0075	1.5028 (1.4415-1.5719)	0.0075 (-0.0143-0.0214)	0.9993
γ- tocopherol	1.0 - 7.0	y = 0.3012x - 0.0168	0.3012 (0.2860-0.3350)	-0.0168 (-0.0517-0.0167)	0.9974
α- tocopherol	1.5 - 40	y = 0.2410x + 0.0174	0.2410 (1.4415-1.5719)	0.0075 (0.2201-0.2784)	0.9982

Table 2: Accuracy and precision of the vitamins analyzed in human serum

Vitamin	Target Values*, µg/mL	Intra- day (n = 4) mean ± SD, µg/mL	% Recovery	CV, %	Inter- day (n = 6) mean ± SD, µg/mL	% Recovery	CV, %
Retinol	0.45	0.419 ± 0.012	93.1	2.91	0.399 ± 0.008	88.6	1.96
	1.5	1.33 ± 0.077	88.7	5.82	1.30 ± 0.051	86.7	3.89
γ- tocopherol	2.0	2.06 ± 0.133	102.9	6.45	2.07 ± 0.054	103.6	2.60
	4.5	5.21 ± 0.332	115.7	6.37	5.27 ± 0.090	117.1	1.71
α- tocopherol	6.0	6.54 ± 0.429	109.0	6.55	6.42 ± 0.211	107.0	3.29
	30	31.1 ± 1.45	103.6	4.67	30.8 ± 0.653	102.5	2.12

*CRM: Vitamin A Plus E Low and High from UTAK Laboratories, Inc. (USA)

Table 3: Results of participation in VITAL-EQA Program of CDC Round 33

	Results (µg/mL) Level 1	Level 2	Level 3
Lab's Overall Mean	0.28	0.538	0.745
Lab's SD	1.42	1.08	4.54
Lab Imprecision (CV)	5.1%	2.0%	6.1%
Imprecision Evaluation	Desirable	Optimum	Minimum
Deviation from Target (%)	-0.1%	-1.5%	-5.7%
Difference Evaluation	Optimum	Optimum	Desirable
	Evaluation Criteria:		
CDC Target Value	0.28	0.545	0.79
CDC Within-Lab SD	1.18	2.36	3.41
Allowable Imprecision	Optimum Performance (%): < 2.6		
	Desirable Performance (%): < 5.2		
	Minimum Performance (%): < 7.8		
	Unacceptable Performance (%): ≥ 7.8		
Allowable Difference	Optimum Performance (%): < ± 4.1		
	Desirable Performance (%): < ± 8.2		
	Minimum Performance (%): < ± 12.3		
	Unacceptable Performance (%): ≥ ± 12.3		

Application of the developed method was confirmed by participation in US Centers for Disease Control and Prevention (CDC)'s Vitamin A Laboratory- External Quality Assurance (VITAL-EQA) Program for the analysis of retinol in human serum. EQA program for analysis of tocopherols in human serum is yet to be participated in. Results from EQA program participation are presented in Table 3. Based on these results, the laboratory achieved an acceptable performance for the three levels of retinol in human serum. Analysis of Level 2 EQA samples achieved optimum performances both for allowable imprecision and allowable difference.

The UPLC method with photodiode array detection was developed and provides a rapid, reliable, precise and specific simultaneous analysis of retinol, γ-tocopherol and α-tocopherol in human serum. The method can be applied to assessment of nutritional status and clinical studies correlating

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these vitamins to different diseases.

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Conflict of interest

The author declares no conflict of interest.

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