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An optimized and validated RP-HPLC/UV detection method for simultaneous determination of all-trans-Retinol (Vitamin A) and α-Tocopherol (Vitamin E) in human serum: Comparison of different particulate reversed-phase HPLC columns

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ABSTRACT

A novel, simple and fast reversed-phase HPLC/UV method was developed, optimized for various chromatographic conditions, and validated according to international guidelines for simultaneous determination of all-trans-retinol and α-tocopherol in human serum using retinyl acetate as internal standard in the concentration of 0.5 μg/ml. A liquid-phase extraction was applied to the 250 μl of serum with n-hexane–dichloromethane mixture (70:30, v/v), in two steps, using ethanol–methanol mixture (95:5, v/v) for protein precipitation and BHT (butylated hydroxy toluene) as stabilizer for sample preparation. Both analytes were analyzed on Kromasil 100 C_{18} column (150 mm × 4.6 mm, 5 μm), Brownlee analytical (Perkin Elmer) C_{18} column (150 mm × 4.6 mm, 5 μm), and Supelco (Supelcosil) LC-18 column (150 mm × 3 mm, 3 μm), protected by a Perkin Elmer C_{18} (30 mm × 4.6 mm, 10 μm; Norwalk, USA) pre-column guard cartridge, at 292 nm wavelength, using methanol–water (99:1, v/v), in isocratic mode as mobile phase applied at flow rate of 1.5 ml/min and 1 ml/min for both 5 μm and 3 μm columns, respectively. Complete separation of all the analytes was achieved in 3 and 6 min on 3 μm and 5 μm columns, respectively by injecting 20 μl of sample into the HPLC system by autosampler, keeping column oven temperature at 25 °C. Different particulate reversed-phase chromatographic columns were evaluated in order to select the best column in terms of sensitivity, selectivity, resolution and short run time of both the analytes and it was concluded that 3 μm columns are better to be used in clinical set up as well as in laboratories for the separation of these analytes in a shorter time as compared with 5 μm columns. The method was validated and applied for the analysis of all-trans-retinol and α-tocopherol in the serum of human volunteers.

1. Introduction

Oxidative stress is defined in general as excess formation and/or insufficient removal of highly reactive molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) [1–3]. Oxidative stress, an imbalance between oxidants and antioxidants in favor of the oxidants potentially leading to damage, is believed to be implicated in the etiopathology of a number of diseases including cancer, atherosclerosis, arthritis, neurodegenerative disorders, coronary heart disease and other conditions [4,5]. However, the power of oxidants to modify molecules in a deleterious fashion is blunted by an array of intracellular and extracellular antioxidants, which may be defined as "substances that when present at low concentrations compared with that of an oxidizable substrate significantly delay or inhibit oxidation of that substrate" [6]. These antioxidants include compounds of both enzymatic and non-enzymatic nature. Among fat-soluble group of vitamins, Vitamin A (all-trans-retinol) and Vitamin E (α-tocopherol) are major components of the antioxidant system in humans, protecting cell membranes against peroxidation [6–10]. Several observational cohort studies described that these vitamins may play a role in reducing the risk of cancer [11], cardiovascular disease [12,13] and neurodegenerative diseases [14], however, clinical trials showed no benefits from the supplementation of these antioxidant vitamins [15,16].

Several methods have been proposed for the measurement of oxidative stress and most of these methods described indirect determinations of the levels of these antioxidants. This is due to the short-lived nature of reactive oxygen/nitrogen species formed and lake of well-established methods for measuring oxidative stress status in humans [17]. Although several methods for the determination of these vitamins in plasma and serum have been

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reported, information on their analytical quality is lacking [18–23]. To solve this discordance the accurate measurement of serum concentration of retinol and α-tocopherol is an important issue [4]. The quantification of these vitamins by LC has been accomplished by normal [24] and reverse-phase liquid chromatography. These methods have employed fixed wavelength or diode-array UV detection [24,25–31], fluorescence detection [24,30–35] and electrochemical detection [24,25,36]. A number of reversed-phase liquid chromatographic (RP-LC) methods have been reported for the determination of all-trans-retinol and α-tocopherol in human body fluids [20,21,26–29,37,38], pharmaceuticals and foodstuffs [39,40]. These fat-soluble vitamins were also determined by capillary electrophoresis, and microemulsion electrokinetic chromatography [41–43]. Recently liquid chromatography with APCI-MS/MS method was applied for the analysis of α-tocopherol and carotenoids in serum [44], and botanical materials [45].

As far as stationary phase is concerned, all the researchers who have adopted reverse-phase liquid chromatographic method have used C_{18} column from different manufacturers. However, the columns used vary in length, particle size, and internal diameter. Although the use of various normal phase columns [30,46,47] and reversed-phase monolithic [48–51] and octyl-modified silica (OS) [46] columns has been reported, reversed-phase conventional particulate octadecylsilane (ODS) columns seem to be the method of choice for the analysis of all-trans-retinol and α-tocopherol in serum/plasma as monolithic columns are expensive in comparison to particulate columns [22,32,52–56]. In a study separation of both the analytes in serum has been achieved on 3 μm column with DAD and fluorescence detector within 13 min; however, comparison of various particulate columns has not been performed [57]. In other reported study gradient elution was performed with Agilent Zorbax Rapid Resolution HT Eclipse plus C_{18} reverse phase 1.8 μm column and UV detection at 325, and 295 nm for the separation of retinol and α-tocopherol in serum, respectively. The complete separation was achieved in 3 min, however, peaks resolution was not so good [58]. Examples of RP-C_{18} columns used are: Zorbax Eclipse XDB-C_{18} column (150 mm × 4.6 mm; 5 μm particle size) [22], NovaPak C_{18} column (150 mm × 3.9 mm; 4 μm bead size) and Whatman Partisphere-5 C_{18} replaceable cartridge (110 mm × 4.7 mm; 5 μm bead size) [33], RP-18e, 100 mm × 4.6 mm monolithic column [48], Hypersil 5ODS 250 mm × 4.6 mm [52], ODS C_{18} column (NovaPack 150 mm × 3.9 mm, 4 μm) [53], analytical scale (25 cm × 0.4 cm) SS Exil ODS column with a particle size 5 μm [54], C_{18}, 250 mm × 3 mm, Nucleosil 5 μm Column [55], Techsphere ODS-2 packed (5 μm particle and 80 å pore size) column (250 mm × 4.6 mm) [56], Water Spherisorb ODS2 (150 mm × 4.6 mm; 3 μm) column [57], Agilent Zorbax C18 RP (50 mm × 4.6 mm; 1.8 μm) column [58].

Based on previous reported work, our developed method is simple, rapid, accurate, reliable, sensitive, selective and cost effective for the determination of all-trans-retinol and α-tocopherol in human serum. The developed method was optimized under various chromatographic conditions and a comparison among different particulate reversed-phase stationary phases (RP-columns of 5 μm and 3 μm particle size), was made. The method was also validated according to standard/internationals guidelines [59]. The optimized validated method was applied for the analysis of all-trans-retinol and α-tocopherol in human serum.

2. Materials and methods

2.1. Materials

DL-α-Tocopherol, purity 97%, all-trans-retinol, purity 97%, retinyl acetate (Vitamin A acetate), α-tocopheryl acetate, ascorbic acid (vitamin C), and hydrochlorothiazide were obtained from Fluka (Sigma–Aldrich, Oslo, Norway). Methanol, acetonitrile, diethyl ether, tetrahydrofuran, chloroform, dichloromethane, n-hexane, ethanol (All solvents of HPLC grade), and 2,6-bis(1,1-dimethylethyl)-4-methylphenol (butylated hydroxyl toluene, BHT), were purchased from Sigma–Aldrich (Oslo, Norway). Purified water was prepared using a Millipore ultra-pure water system (Milford, USA).

2.2. Preparation of standard solutions

Stock solutions of all-trans-retinol (1 mg/ml), α-tocopherol (1 mg/ml) and internal standards (retinyl acetate, ascorbic acid, and hydrochlorothiazide), 1 mg/ml were prepared in methanol and stored in amber glass vials at −20 °C until analysis. Working solutions for both standards were made in volumetric flasks (10 ml) using methanol:water (99:1, v/v) in the concentration range of 0.05–4 μg/ml, and 0.2–10 μg/ml for all-trans-retinol and α-tocopherol, respectively. The concentration of internal standard in standard solutions was kept constant (0.5 μg/ml) by making its dilution with methanol. The calibration curves were obtained at eight concentration levels for both all-trans-retinol and α-tocopherol standard solutions. Similarly, a 1:1 mixture containing 1 μg/ml each of all-trans-retinol, α-tocopherol and internal standard were prepared in methanol.

2.3. Sample preparation

Blood samples collected in borosilicate glass tubes were centrifuged at 1600 × *g* for 10 min at 0 °C to obtain serum. The serum was then stored at −80 °C until analysis. For sample preparation the serum was thawed and spiked with the all-trans-retinol and α-tocopherol standard solutions. A 12.5 μl internal standard solution (10 μg/ml stock solution) was added to serum keeping its concentration 0.5 μg/ml in the final dilution and vortexed for 1 min. The following liquid–liquid extraction procedure was applied. Serum (250 μl) was transferred to glass vials with screw cap and deproteinized with ethanol–methanol (95:5, v/v), mixture (750 μl). Then centrifuged for 5 min at 1600 × *g* and 0 °C. Extraction was carried out with 1000 μl mixture of n-hexane-dichloromethane (70:30, v/v), containing 15 μg/ml BHT by vortexing for 5 min followed by centrifugation at 1600 × *g* for 10 min at 0 °C. The extraction process was repeated twice. The clear supernatant was transferred to a borosilicate glass tube and evaporated under nitrogen. The residues were dissolved in methanol (250 μl), vortexed for 1 min and 20 μl of this extracted serum was injected into HPLC for analysis. Since, both the vitamins are light and heat sensitive, antioxidants such as ascorbic acid and BHT were incorporated to the samples, during sample preparation as stabilizers, and the process of sample preparation and handling was carried out in dim light and at room temperature (25 °C). Calibration curves were constructed for the all-trans-retinol and α-tocopherol using retinyl acetate (0.5 μg/ml) as internal standard in mobile phase, spiked serum, as well as spiked serum corrected for blank serum, on Kromasil 100 C_{18} column (150 mm × 4.6 mm, 5 μm), Brownlee analytical (Perkin Elmer) C_{18} column (150 mm × 4.6 mm, 5 μm), and Supelco™ LC-18 column (150 mm × 3 mm, 3 μm).

2.4. Methods

The study was carried out using a Perkin Elmer HPLC system (Norwalk, USA) consisted of a pump (series 200), on-line vacuum degasser (series 200), autosampler (series 200), column oven (series 200), linked by a Pe Nelson network chromatography interface (NCI) 900 with a UV/VIS detector (series 200). The whole HPLC system was controlled by Perkin Elmer Total Chrom

Workstation Software (version 6.3.1). The data was acquired and quantified by this software. The analysis was performed using different particulate reversed-phase chromatographic columns; Kromasil 100 C_{18} column (150 mm × 4.6 mm, 5 μm; Thames Restek, UK), Brownlee analytical C_{18} column (150 mm × 4.6 mm, 5 μm; Perkin Elmer, Shelton, USA), and Supelco™ (Supelcosil) LC-18 column (150 mm × 3 mm, 3 μm; Bellefonte, USA), protected by a Perkin Elmer C_{18} (30 mm × 4.6 mm, 10 μm; Norwalk, USA) pre-column guard cartridge.

2.5. Chromatographic conditions

The chromatography was performed using different particulate reversed-phase chromatographic columns mentioned in Section 2.4 (Kromasil 100 C_{18}, Brownlee analytical C_{18}, Supelco™ LC-18) applying various organic solvents including methanol, acetonitrile, tetrahydrofuran, and water in different compositions as mobile phase, in isocratic mode, at flow rate of 0.8–2 ml/min keeping column oven temperature in the range of 25–50 °C. The autosampler loop volume was kept in the range of 10–50 μl. Detector wavelength was varied in the range of 280–300 nm for the simultaneous determination of all-trans-retinol and α-tocopherol applying retinyl acetate, tocopheryl acetate, ascorbic acid and hydrochlorothiazide as internal standard.

2.5.1. Comparison of columns

To choose the most stable, specific, reproducible and high performance stationary phase for developing a rugged, reproducible and optimized HPLC method various reverse-phase columns were tested and evaluated in terms of separation, retention, resolution, efficiency, selectivity, peak tailing and back-pressure values. A comparison was performed among different particulate columns of 3 μm and 5 μm particle size from different suppliers. For this purpose, different reversed-phase particulate chromatographic columns including Kromasil 100 C_{18} column (150 mm × 4.6 mm, 5 μm; Thames Restek, UK), Brownlee analytical C_{18} column (150 mm × 4.6 mm, 5 μm; Perkin Elmer, Shelton, USA), Supelco™ (Supelcosil) LC-18 column (150 mm × 3 mm, 3 μm; Bellefonte, USA), were studied.

2.6. Validation of the method

The goal of the validation process is to challenge the method under various HPLC parameters and determine limits of allowed variability for the various conditions needed to run the method. The precision, specificity, sensitivity, linearity, recovery, detection and quantitation limits, robustness, stability of solutions and system suitability parameters were evaluated [59]. The developed method was validated by applying the following parameters.

The specificity of the developed chromatographic method was evaluated by determining the separation of both fat-soluble antioxidants vitamins in mobile phase, blank serum, and 1:1 mixture containing 1 μg/ml each of both analytes and spiked serum samples with 1 and 4 μg/ml standard mixtures of all-trans-retinol and α-tocopherol, respectively.

The accuracy of the method was measured by recovery method. The % recovery was calculated at three nominal concentrations by spiking the serum (250 ml) with 0.8, 1 and 2 μg/ml of each analyte, keeping internal standard concentration 0.5 μg/ml, extracting with the above procedure mentioned in Section 2.3 and injecting 20 μl sample into HPLC system in triplicate. Recovery was determined according to the following equation:

$$\text{Recovery} = \frac{[C] \times 100}{[A] + [B]} \quad (1)$$

where [A] = peak area response ratios of the analytes with reference to internal standard in the mobile phase; [B] = peak area response ratios of analytes with reference to internal standard in the control serum; [C] = peak area response ratios of the analytes with reference to internal standard in spiked serum.

The linearity of the method was assessed from the calibration curves obtained at eight concentration levels of both all-trans-retinol and α-tocopherol. Calibration curves were constructed for all-trans-retinol and α-tocopherol in the mobile phase and spiked serum by plotting the response ratios (ratios of peak areas of analytes to internal standard) versus spiked concentration of all-trans-retinol and α-tocopherol using a linear least squares regression. The resulting plot slope (m), intercept (b), correlation coefficient (r) and standard error (E_s) were calculated from the regression equation using Microsoft Excel 2007 installed on PC.

Precision study was carried out on the basis of injection repeatability and analysis repeatability of spiked serum samples. Injection repeatability was determined by spiking serum sample with 1 μg/ml each of all-trans-retinol and α-tocopherol and injecting 10 times into the HPLC system. The retention time and peak area repeatability data obtained as mean, standard deviation (SD) and covariance (% RSD), was expressed as a measure of precision of the method. Analysis repeatability was measured by analyzing five serum samples spiked with 1 μg/ml of each analyte, prepared individually from one human serum and the results were obtained as repeatability of recovered amount, expressed by mean, standard deviation (SD), and covariance (% RSD). For the determination of intermediate precision intra-day and inter-day study was performed on spiked serum samples at 8:00, 16:00, and 24:00 h, for 3 consecutive days. The results were obtained in the form of recovered amount and expressed as mean, standard deviation (SD), and covariance (% RSD) calculated from the resulted data. The recovered amount was calculated in the form of concentration by the following equation:

$$C = \left(\frac{X}{Y}\right) \times \left(\frac{A}{B}\right) \times C_s \times F_D \quad (2)$$

where X and Y are peak areas of the analyte in serum samples and 1:1 mixture (1 μg/ml of each all-trans retinol, α-tocopherol and internal standard), respectively; A and B are peak areas of the internal standard in 1:1 mixture (1 μg/ml of each all-trans-retinol, α-tocopherol and internal standard) and serum samples, respectively; C_s is the concentration of analyte in the 1:1 mixture; and F_D is the dilution factor.

The sensitivity of the method was evaluated by quantifying the limit of detection (LOD) and limit of quantification (LOQ) for all-trans-retinol retinol and α-tocopherol. The limit of detection (LOD) of a compound is the concentration at which signal-to-noise ratio (S/N) is three and limit of quantification (LOQ) is the concentration of compound that produced a response equal to 10 times the value of signal-to-noise ratio (S/N). For LOD and LOQ quantification dilutions were prepared in the ranges of 0.5–5 ng/ml and 5–20 ng/ml for all-trans-retinol and 1–50 ng/ml and 10–100 ng/ml for α-tocopherol, respectively. The LOD and LOQ were then determined from the peaks by the software at signal-to-noise ratio (S/N) of three and ten, respectively.

The robustness/ruggedness of the reported method was determined by bringing small deliberate changes in the various chromatographic conditions, like mobile phase composition (±2%), column oven temperature (±5 °C), detector wave length (±2 nm) and flow rate of mobile phase (0.2 ml/min).

Stability study of spiked serum samples stored at 25 °C, 4 °C and −20 °C and standard stock solutions stored at 25 °C and 4 °C was carried out for 3 days, and 1 month, respectively. The % stability

was calculated by the following equation:

$$\%\,\text{Stability} = \frac{S_t}{S_0} \times 100 \quad (3)$$

where S_t is stability of analyte at time t, and S_0 is stability at initial time.

Various parameters such as the retention factor (k), separation factor (α), tailing factor (T), resolution (R_s), and efficiency (N), were calculated for evaluation of different reversed-phase particulate chromatographic columns and chromatographic system.

3. Results and discussion

The suggested method is simple, accurate, rapid, and novel (comparison of different particulate columns, simultaneous determination of all-trans retinol and α-tocopherol at the same wavelength of UV detector, mobile phase composition, temperature effect, stability study, and optimization of other chromatographic parameters) as compared with the previous reported methods [4,50,51,57,58]. The method was validated for simultaneous determination of all-trans-retinol and α-tocopherol in human serum and various chromatographic conditions and other experimental parameters such as mobile phase composition, stationary phase selection, detector wavelength, flow rate, column oven temperature, internal standard selection and sample preparation were optimized. The mobile phase was selected using water, methanol, tetrahydrofuran and acetonitrile in different proportions. Pure methanol (100%) showed best result for the separation of all-trans-retinol and α-tocopherol standard mixtures; however, in spiked serum samples separation was poor. Variation in the mobile phase composition through incorporation of 1–10% water was made and complete separation of all the peaks was obtained with the optimized mobile phase composition 99% methanol in water as shown in Table 1 and Fig. 1.

3.1. Comparison of different columns

The stationary phase was selected through comparison of various particulate octadecylsilane (ODS) and octasilane (OS) reversed-phase columns. Among all these stationary phases the better resolution and sensitivity was obtained with particulate octadecylsilane (ODS) reversed-phase stationary phase in comparison with octasilane (OS) stationary phases. Three particulate reversed-phase columns; Kromasil 100 C_{18} column (150 mm × 4.6 mm, 5 μm), Brownlee analytical C_{18} column (150 mm × 4.6 mm, 5 μm), Supelco™ (Supelcosil) LC-18 column (150 mm × 3 mm, 3 μm) of different manufactures having different particle size were compared on the basis of accuracy, precision, separation, retention, resolution, efficiency, selectivity, peak tailing, back-pressure of column and mobile phase consumption, in order to achieve rapid, sensitive and cost effective stationary phase. The observed validation values were similar for all the particulate columns, except retention factor, separation factor, tailing factor, peak resolution, efficiency of column, back-pressure of column, retention time and peak shape of analytes and mobile phase consumption, differed as shown in Table 2. The slope and intercept values calculated from the calibration curves of all-trans-retinol and α-tocopherol on Supelcosil column were higher than the values obtained on the others columns as shown in Table 3. Variation in the serum concentration of all-trans-retinol and α-tocopherol obtained on all the three columns found nonsignificant applying Tukey test (ANOVA), as shown in Table 4. Although, rapid analysis and best sensitivity corresponding to all-trans-retinol (peak A) at 1.25 min, retinyl acetate (peak B) at 1.5 min, and α-tocopherol (peak C), at 2.61 min, was obtained with Supelcosil column, Fig. 2 and Table 3, it developed high back-pressure of 3410–3450 psi with spiked samples, as compared to the back-pressure of other columns ranging from 2240 to 2290 psi (Table 2). The pressure of less than 50% of the capability of the pump is required for optimum system reliability and column life. The retention time and mobile phase consumption were reduced by two times using Supelcosil 3 μm column as compared to Kromasil and Perkin Elmer 5 μm columns. After analyzing a large number (more than 250 serum samples) of serum samples peak tailing and longer retention time was observed for both analytes on Supelcosil column as compared to the other columns where only minor changes in peak shape and retention times were observed. The 3 μm particle size column is better for fast analysis, but has short shelf-life and more prone to plug, sampling problems and instrumental peak broadening effect as compared to 5 μm particle size columns.

The mobile phase flow rates in the range of 0.8–2 ml/min were applied to show the effect of flow rates on the separation and resolution of fat-soluble antioxidants (all-trans-retinol and α-tocopherol). The optimum flow rate of 1.5 ml/min for Kromasil and Perkin Elmer and 1 ml/min for Supelcosil column was selected because the lower flow rates resulted in longer retention times and poor resolution of the analytes while the higher flow rates showed higher back-pressure especially with serum samples and 3 μm particle size column (Supelcosil column).

The wavelength was selected for simultaneous analysis of all-trans-retinol and α-tocopherol by monitoring the column eluent through detector at different wavelengths, in the range of 280–300 nm. Based on our literature knowledge of the physicochemical properties of these vitamins, the wavelengths most commonly used for simultaneous determination of all-trans-retinol and α-tocopherol, their sensitivity to UV detector (molar absorptivity coefficients of both vitamins), and on the results obtained in our laboratory, 292 nm is the optimum UV-detector wavelength for simultaneous determination of all-trans-retinol and α-tocopherol. Above this wavelength, the sensitivity of Vitamin A increased while that of α-tocopherol decreased and below this wavelength, the situation was vice versa.

Selection of column oven temperature was based on better sensitivity, good resolution peaks and shorter retention times of both analytes (all-trans-retinol and α-tocopherol). Column oven temperature variation was brought in the range of 25–50 °C, by increment of 5 °C. Better results were obtained with temperature variation between 30 and 35 °C. Higher temperature resulted in shorter retention times, but poor sensitivity (low peak response and tailing) and peak resolution of all-trans-retinol, retinyl acetate and α-tocopherol, while lower temperatures resulted in longer retention times and broader peaks of all the analytes (Fig. 3).

Internal standard was chosen on the basis of its sensitivity, specificity, stability and compatibility with others analytes and extraction procedure. Retinyl acetate, tocopherol acetate, ascorbic acid and hydrochlorothiazide were tested as internal standard to minimize the variability in the extraction procedure. Among all these compounds retinyl acetate showed the best results to be chosen as internal standard. Although tocopherol acetate was more stable than retinyl acetate it has longer retention time, low recovery with extraction solvent, and less sensitivity as compared to retinyl acetate. Ascorbic acid showed less stability and hydrochlorothiazide showed minimum recovery with the selected extraction solvents.

3.2. Sample preparation

Various organic solvents were used for the preparation of stock solutions of all-trans-retinol, retinyl acetate and α-tocopherol. Methanol was selected due to greater solubility of all the analytes in methanol as compared to other organic solvents. Working solutions were prepared by diluting the stock solutions of all-

Table 1
Separation of all-trans retinol and alpha-tocopherol using various solvents in different composition as a mobile phase.

Mobile phases	All-trans retinol		Retinyl acetate		Alpha-tocopherol	
	Rt[a]	*PA*[b]	*Rt*	*PA*	*Rt*	*PA*
Methanol (100%)	2.04	83,237	2.76	85,846	5.67	65,567
Methanol:water (99:01)	2.10	78,248	2.87	80,513	6.08	62,353
Methanol:water (95:05)	2.14	70,138	3.12	68,468	8.88	53,436
Methanol:water (90:10)	3.58	70,550	6.40	45,393	11.56	19,498
Methanol:acetonitrile:water (65:30:5)	2.49	64,153	3.47	63,213	8.00	54,421
Methanol:acetonitrile:tetrahydrofuran (75:25:05)	2.22	65,989	3.81	65,370	11.19	53,886

[a] Retention time of analyte.
[b] Peak area of analyte.

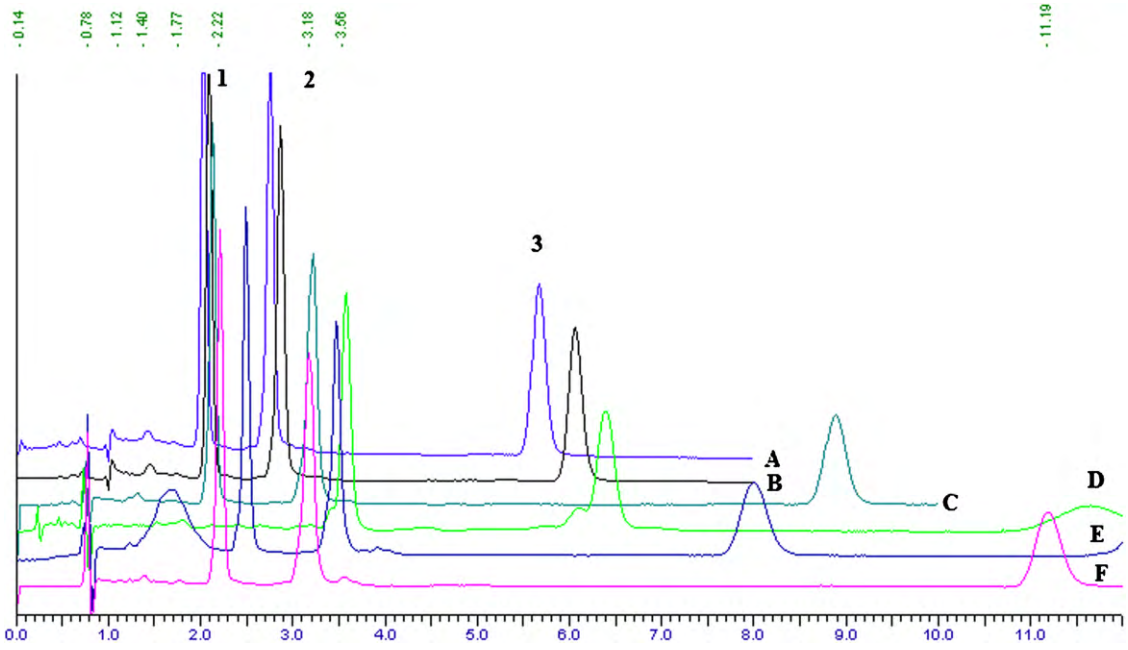

Fig. 1. HPLC operation program showing various solvents in different compositions as a mobile phase.

trans-retinol and α-tocopherol with methanol–water (99:01, v/v) in volumetric flasks. A two-step liquid-phase extraction was carried out for the recoveries of all-trans-retinol and α-tocopherol from serum, using various organic solvents. Maximum recoveries of all analytes reported with n-hexane–dichloromethane (70:30, v/v) mixture. Although, the sample preparation was carried out in dim light and at controlled temperature, various stabilizers were added to avoid the oxidation of antioxidant vitamins in the presence of atmospheric oxygen and to increase their stability and storage capability. For this purpose, ascorbic acid and BHT incor-

Table 2
Comparison of columns specifications and system suitability tests.

Specification and tests	Columns		
	Kromasil	Brownlee (Perkin Elmer)	Supelcosil
Internal diameter (mm)	4.6	4.6	3
Particle size (μm)	5	5	3
Back-pressure (psi)	2275–2290	2240–2290	3410–3450

Specification and tests	Columns									Means ± SD		
	Kromasil			Brownlee (Perkin Elmer)			Supelcosil			A	IS	E
	A[a]	IS[b]	E[c]	A	IS	E	A	IS	E			
Retention factor, *k*	0.9	1.53	4.15	0.7	1.22	3.45	0.47	0.75	1.89	0.69 ± 0.22	1.17 ± 0.39	3.16 ± 1.16
Separation factor, *α*	1.45	1.7	2.71	1.6	1.74	2.84	2.05	1.59	2.53	1.70 ± 0.31	1.67 ± 0.08	2.69 ± 0.15
Tailing factor, *T*	1.09	0.92	0.96	1.05	1.05	1.08	1.3	1.55	1.39	1.15 ± 0.13	1.17 ± 0.33	1.14 ± 0.22
Resolution, R_s	2.45	3.72	8.96	3.03	4.87	12.6	2.05	2.1	7.33	2.51 ± 0.49	3.56 ± 1.39	9.63 ± 2.69
Efficiency or number of theoretical plates, *N*	3125	2463	2902	5759	5154	5912	4072	3346	5827	4319 ± 1334	3654 ± 1372	4880 ± 1714

[a] All-trans retinol.
[b] Retinyl acetate.
[c] α-Tocopherol.

Table 3
Concentration range, linearity, accuracy, repeatability, and sensitivity of the developed method.

S/No.	Parameters	Kromasil		Brownlee (Perkin Elmer)		Supelcosil	
		All-trans retinol	α-Tocopherol	All-trans retinol	α-Tocopherol	All-trans retinol	α-Tocopherol
(1)	Concentration range (μg/ml)	0.05–4	0.2–10	0.05–4	0.2–10	0.05–4	0.2–10
(2)	Linearity						
(a)	Standard solutions						
	Regression Equation	$y = 0.85x + 0.05$	$y = 0.33x + 0.08$	$y = 0.95x + 0.08$	$y = 0.43x + 0.03$	$y = 1.31x + 0.13$	$y = 0.61x + 0.10$
	Correlation coefficient, r	0.999	0.999	0.999	0.999	0.998	0.999
	Standard error, E_s	0.0321	0.0428	0.0425	0.0245	0.0657	0.0586
(b)	Spiked serum samples						
	Regression equation	$y = 0.86x + 0.09$	$y = 0.35x + 0.13$	$y = 0.98 + 0.15$	$y = 0.44x + 0.13$	$y = 1.32x + 0.15$	$y = 0.61x + 0.18$
	Correlation coefficient, r	0.999	0.999	0.999	0.999	0.999	0.999
	Standard error, E_s	0.0302	0.0406	0.0386	0.0219	0.0343	0.054
(c)	Corrected serum samples						
	Regression equation	$y = 0.86x + 0.04$	$y = 0.35x + 0.07$	$y = 0.98x + 0.07$	$y = 0.44x + 0.03$	$y = 1.32x + 0.08$	$y = 0.61x + 0.09$
	Correlation coefficient, r	0.999	0.999	0.999	0.999	0.999	0.999
	Standard error, E_s	0.0302	0.0406	0.0386	0.0219	0.0343	0.054
3	Accuracy (% recovery)						
	0.8 μg/ml	96.62 ± 1.84; 1.91	98.66 ± 2.38; 2.41	97.26 ± 1.86; 1.91	98.42 ± 2.57; 2.61	99.32 ± 1.10; 1.11	98.36 ± 1.70; 1.19
	1.0 μg/ml	95.40 ± 5.37; 5.62	96.23 ± 1.27; 1.32	96.70 ± 2.87; 2.97	95.65 ± 4.55; 4.76	99.49 ± 2.30; 2.31	99.34 ± 1.28; 1.29
	2.0 μg/ml	97.33 ± 1.40; 1.44	101.38 ± 5.21; 5.14	97.33 ± 1.40; 1.44	98.00 ± 2.84; 2.87	99.50 ± 0.81; 0.81	98.09 ± 0.98; 0.99
4	Repeatability						
(a)	Injection repeatability						
	Retention time (min)	1.91 ± 0.01; 0.35	4.94 ± 0.030; 0.59	1.85 ± 0.01; 0.38	4.85 ± 0.01; 0.16	1.25 ± 0.01; 0.53	2.61 ± 0.01; 0.30
	Peak area	30,302 ± 478; 1.58	58,525 ± 388; 0.66	32,658 ± 385; 1.18	59,261 ± 261; 0.44	44,468 ± 336; 0.76	88,844 ± 165; 0.19
(b)	Analysis repeatability						
	Amount recovered (μg/ml)	0.97 ± 0.02; 1.91	0.96 ± 0.03; 3.35	0.98 ± 0.20; 2.00	0.99 ± 0.01; 0.69	0.99 ± 0.01; 0.84	0.98 ± 0.01; 0.59
5	Sensitivity						
(a)	Limit of detection (ng/ml)	3	29	2	25	1	5
(b)	Limit of quantification (ng/ml)	10	90	7	79	3.5	18

Table 4
Concentration of analytes determined in human volunteers ($n = 15$) on various columns.

Analytes	Columns								
	Kromasil			Brownlee (Perkin Elmer)			Supelcosil		
	Mean ± SD	Minimum	Maximum	Mean ± SD	Minimum	Maximum	Mean ± SD	Minimum	Maximum
All-trans-retinol (μg/ml)	0.5409 ± 0.2150	0.2537	0.8563	0.5632 ± 0.2107	0.2998	0.9049	0.5789 ± 0.2115	0.3297	0.9121
α-Tocopherol (μg/ml)	3.8643 ± 2.1923	1.2280	8.0526	4.060 ± 2.2135	1.2289	8.2280	4.1246 ± 2.1523	1.2680	8.3869

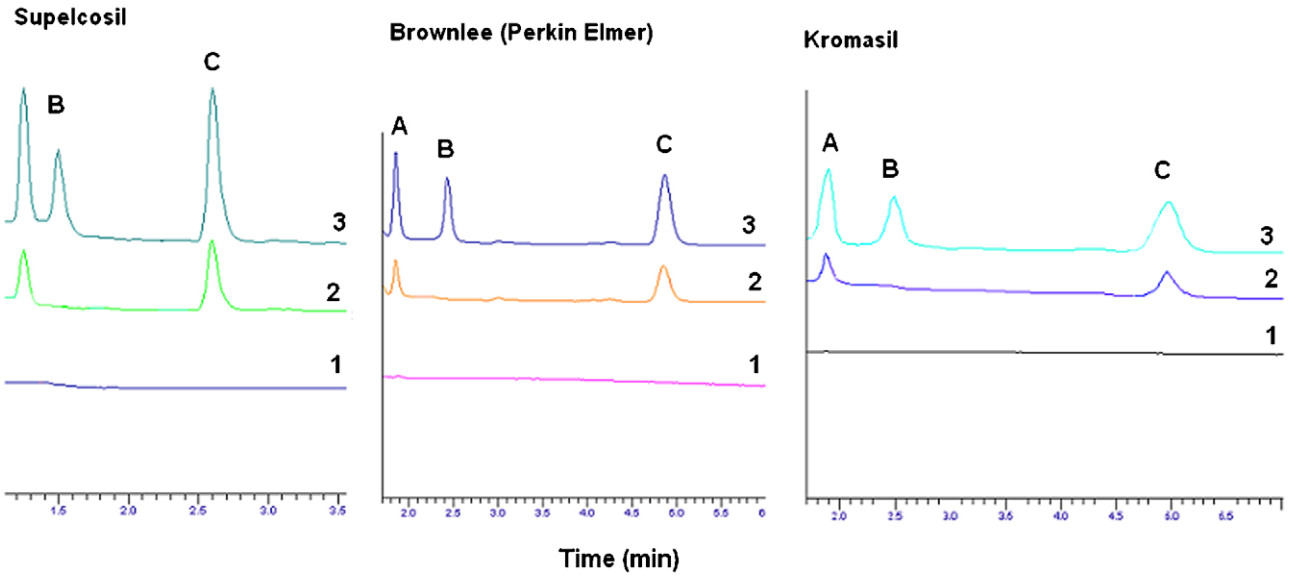

Fig. 2. Representative RP-HPLC chromatograms of various samples analyzed under specified conditions on different columns.

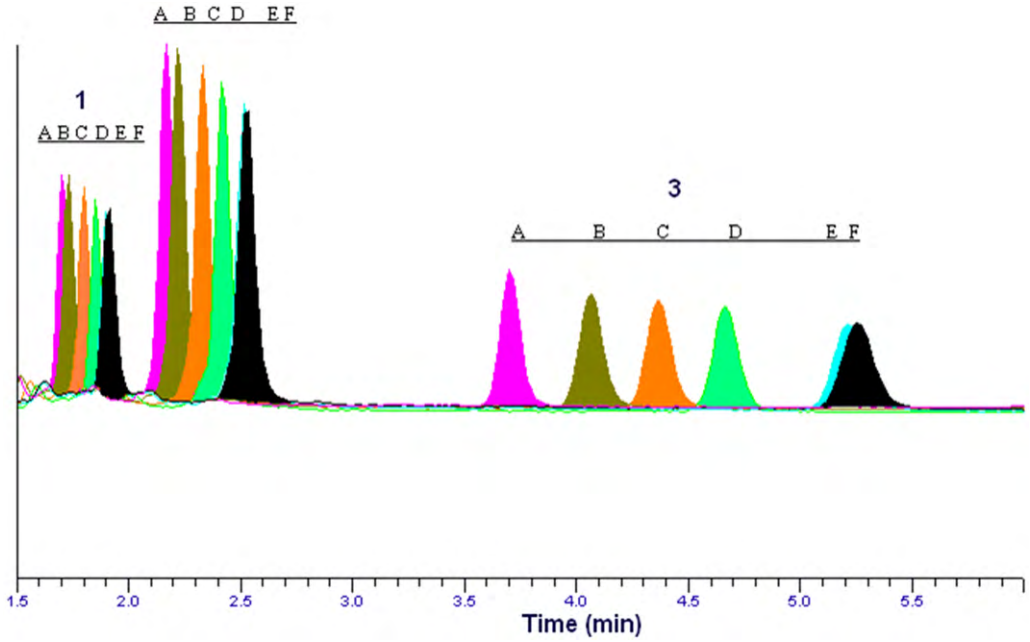

Fig. 3. Effect of column oven temperature on the analysis of all-trans-retinol (peaks 1) and α-tocopherol (peaks 3). Peaks: A at 50 °C; B at 45 °C; C at 40 °C; D at 35 °C; E at 30 °C; F at 25 °C.

porated in protein denaturing solvents and extraction solvents in different concentration ranges were added to the serum samples. Better results were obtained with BHT in the concentration range of 5–15 μg/ml in terms of stability, peaks shape resolution, and analytes recoveries as compared to the results obtained with ascorbic acid. The concentration of BTH was optimized, as higher concentration of BHT resulted in large interfering peak that suppressed the analytes peaks in chromatogram. During extraction process complete protein precipitation was achieved with a mixture of ethanol–methanol (95:5, v/v), at least three times the volume of serum. The organic layer was evaporated under gaseous nitrogen to avoid the degradation of analytes and residue was reconstituted with 250 μl methanol–water (99:01, v/v), keeping dilution factor one.

3.3. Method validation

The developed method was validated by applying international guidelines [59]. The precision, specificity, sensitivity, linearity, recovery, detection and quantitation limits, robustness, stability of solutions and system suitability parameters were evaluated. The laboratory results showed that the method was accurate and fully validated for the simultaneous determination of all-trans-retinol and α-tocopherol in serum. Complete resolution of the entire target peaks was observed in 6 min, by analyzing the standard solutions, blank serum and spiked serum samples under this optimized chromatographic method for simultaneous analysis of all-trans-retinol and α-tocopherol.

The calibration curves of standard solutions and spiked serum samples constructed for both antioxidant vitamins on all the three columns showed good linearity in the range of 0.05–4 μg/ml and 0.2–10 μg/ml for all-trans-retinol and α-tocopherol, respectively. Regression equation and correlation coefficient (r) calculated from the calibration curves of standard solutions, spiked serum samples, and spiked serum samples corrected for blank serum for all-trans-retinol were $y = 0.85x + 0.05$, $r = 0.999$; $y = 0.86x + 0.09$, $r = 0.999$; $y = 0.86x + 0.04$, $r = 0.999$; $y = 0.95x + 0.08$, $r = 0.999$; $y = 0.98x + 0.15$, $r = 0.999$; $y = 0.98x + 0.07$, $r = 0.999$; and $y = 1.31x + 0.13$, $r = 0.998$; $y = 1.32x + 0.15$, $r = 0.999$; $y = 1.32x + 0.08$, $r = 0.999$; and for α-tocopherol were $y = 0.33x + 0.08$, $r = 0.999$; $y = 0.35x + 0.13$, $r = 0.999$; $y = 0.35x + 0.07$, $r = 0.999$; $y = 0.43x + 0.03$, $r = 0.999$; $y = 0.44x + 0.13$, $r = 0.999$; $y = 0.44x + 0.03$, $r = 0.999$; and $y = 0.61x + 0.10$, $r = 0.999$; $y = 0.61x + 0.18$, $r = 0.999$; $y = 61x + 0.09$, $r = 0.999$ on Kromasil, Perkin Elmer, and Supelcosil columns, respectively, Table 3. Similarly, the standard error (E_S) calculated for standard solutions, spiked serum and spiked serum samples corrected for blank serum, of all-trans-retinol were 0.0321, 0.0302, 0.0302, 0.0425, 0.0386, 0.0386, 0.0657, 0.0343, 0.0343 and for α-tocopherol were 0.0428, 0.0406, 0.0406, 0.0245, 0.0219, 0.0219, 0.0586, 0.0540, 0.0540 on Kromasil, Perkin Elmer, and Supelcosil columns, respectively, as shown in Table 3.

Accuracy of the method determined on the basis of percent recovery, at 0.8, 1 and 2 μg/ml concentration, for all-trans-retinol, was 96.6, 95.4, 97.3, 97.3, 96.7, 97.3, 99.3, 99.5, 99.5 and that for α-tocopherol, was 98.7, 96.2, 101.4, 98.4, 95.6, 98.0, 98.4, 99.3, 98.0 on Kromasil, Perkin Elmer, and Supelcosil columns, respectively, as shown in Table 3.

The precision data obtained by injection repeatability, analysis repeatability, and intra-day, inter-day study is presented in Tables 3 and 5, respectively. The intra-day coefficients of variation (% CV) for all-trans-retinol at 0.05, 0.2, and 1.2 μg/ml were 1.1, 0.7, 1.6, and for α-tocopherol at 0.5, 2, and 10 μg/ml were 1.1, 0.8, and 1.5%, respectively ($n = 5$). Similarly the corresponding values for inter-day analysis were 3.8, 1.6, and 1.8% for all-trans-retinol and 3.6, 2.2, and 1.5% for α-tocopherol, respectively (Table 5).

Sensitivity of the method determined on the basis of quantification of LOD and LOQ values of all-trans-retinol and α-tocopherol on all the three columns was presented in Table 3. The sensitivity of Supelcosil column was higher than the other two columns.

The results obtained from the stability study showed that the standard solutions would be stable for at least 1 month, if stored at −20 °C. However, spiked serum samples were stable for 24 h when stored at −20 °C. Both standard solutions and spiked serum sample were degraded at room temperature. Among all these analytes retinyl acetate was the most unstable at room (25 °C), as well as freezer temperature (−20 °C).

Method robustness evaluated by bringing minor changes in different chromatographic conditions such as mobile phase

Table 5
Intra-day and inter-day studies performed on Perkin Elmer column.

S/No.	Parameters	Vitamin A (all-trans retinol) Mean ± SD; % CV	Vitamin E (α-tocopherol) Mean ± SD; % CV
(1)	Intra-day repeatability		
	Vit A: 0.05 μg/ml; Vit E: 0.5 μg/ml (amount recovered, μg/ml)	0.0490 ± 0.0005; 1.1101	0.4810 ± 0.0055; 1.1391
	Vit A: 0.2 μg/ml; Vit E: 2 μg/ml (amount recovered, μg/ml)	0.1962 ± 0.0013; 0.6686	1.9233 ± 0.0165; 0.8563
	Vit A: 1.2 μg/ml; Vit E: 10 μg/ml (amount recovered, μg/ml)	1.1818 ± 0.0187; 1.5845	9.87 ± 0.1445; 1.4637
(2)	Inter-day repeatability		
	Vit A: 0.05 μg/ml; Vit E: 0.5 μg/ml (amount recovered, μg/ml)	0.0474 ± 0.0018; 3.8091	0.4809 ± 0.0171; 3.5560
	Vit A: 0.2 μg/ml; Vit E: 2 μg/ml (amount recovered, μg/ml)	0.1960 ± 0.0031; 1.5756	1.9039 ± 0.0413; 2.1705
	Vit A: 1.2 μg/ml; Vit E: 10 μg/ml (amount recovered, μg/ml)	1.1835 ± 0.0208; 1.7596	9.7362 ± 0.1488; 1.5291

composition, column oven temperature, flow rate and detector wavelength, resulted negligible changes in the peak area and retention time of the analytes.

Results of system suitability parameters obtained on different chromatographic columns, presented in Table 2, were in the permissible range [59].

4. Application of the method

Our developed and validated HPLC method was applied for the assessment of oxidative stress through measuring serum concentration of all-trans-retinol and α-tocopherol in healthy volunteers and will be applied for monitoring serum/plasma concentration of these antioxidants in patients with diabetes and cardiovascular diseases. This method is a part of biochemical analysis of blood samples collected from healthy volunteers and patients with diabetes and cardiovascular diseases. Some practical examples are given in Table 4, where this method is utilized for the determination of all-trans-retinol and α-tocopherol in the clinical practice (comparison of different particulate columns). Our data obtained from healthy volunteers showed best peaks resolution, and the serum concentrations of all-trans-retinol and α-tocopherol were within the normal range. We also applied this method for the analysis of cosmetic products (results not shown). This method can also be applied for the investigation of all-trans-retinol and α-tocopherol in foods, food supplements, pharmaceutical preparations and others complex biological matrices if suitable modifications are made in the extraction procedure used for these compounds.

5. Conclusion

The reported optimized and validated HPLC method for the simultaneous determination of all-trans-retinol and α-tocopherol in human serum was simple, economical, rapid, accurate, sensitive, precise, selective and reproducible. The method was optimized using various chromatographic parameters and validated according to standard guidelines [59]. Various chromatographic conditions and experimental parameters were evaluated and selected on the basis of trials. The selected method was also validated on the basis of specificity, sensitivity, linearity, stability, precision, recovery, robustness and system suitability. The comparison of different particulate columns was made to facilitate the rapid, accurate and inexpensive use of this method for more practical work in clinical setup. Serum concentration of all-trans-retinol and α-tocopherol was determined on all the three columns in human volunteers. Separation of both the analytes will be achieved using 3 μm column with higher sensitivity, less solvent consumption and shorter run time as compared with 5 μm columns. The cost of the method can be reduced by analyzing the target compounds in 3 min, using 3 μm column. Our developed method will be applied for the assessment of oxidative stress by monitoring the concentration of all-trans-retinol and α-tocopherol in serum/plasma of healthy volunteers and patients with diabetes and cardiovascular diseases in clinical studies. This method can also be used for the investigation of all-trans-retinol and α-tocopherol in pharmaceutical preparations, food supplement, fortified drinks and biological matrices at relatively low cost if suitable modifications are made in the extraction procedures used for the recovery of these compounds.

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