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Short communication

Rapid quantitative determination of fat-soluble vitamins and coenzyme Q-10 in human serum by reversed phase ultra-high pressure liquid chromatography with UV detection

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ABSTRACT

We are presenting the first ultra-high pressure LC (UHPLC) method for rapid quantitative measurement of vitamin A, E (α- and γ-tocopherol), β-carotene and CoQ_{10} from human serum. The chromatography was performed on Shield RP_{18} UHPLC column with UV detection. The method was validated based on linearity, accuracy, matrix effects study, precision and stability. The calibration was linear over the following range: 0.09–10.0 for retinol and γ-tocopherol, 0.05–5 for β-carotene, 0.9–100 for α-tocopherol and 0.14–15 mg/L for CoQ_{10}. The limit of detection and quantitation for retinol, γ-tocopherol, β-carotene, α-tocopherol and CoQ_{10} were as follows 0.07/0.024, 0.018/0.06, 0.004/0.12, 0.078/0.261, 0.008/0.028 mg/L. The recoveries were above 85%. The inter- and intra-assay precision was below 10%. Reference intervals were established for children and adults. Because of its low cost, extremely short analysis time (2 min) and excellent chromatographic reproducibility this UHPLC method can easily be adopted for high-throughput clinical and pharmacokinetics studies.

1. Introduction

Adequate dietary intake of nutrients such as vitamin A (retinol), E and carotenoids (such as β-carotene–vitamin A precursor) is essential for normal development and health maintenance. Vitamin E (comprised of a group of eight tocopherols and tocotrienols) is the major and most potent lipid soluble antioxidant in vivo. Acting as radical scavenger vitamin E (α- and γ-tocopherol) has been reported to interrupt the lipid peroxidation [1]. Similarly vitamin A reacts indirectly with active oxygen species to inhibit free radical synthesis by increasing the activities of detoxifying systems. Another major lipid peroxidation inhibitor is coenzyme Q (CoQ_{10} or ubiquinone), which also plays an important role in the mitochondrial electron-transfer. Because of their powerful antioxidants potential, carotenoids, retinol, tocopherols and coenzyme Q have been linked with prevention of serious diseases, e.g., cardiovascular, eye diseases, atherosclerosis, bone calcification, neurological disorders (e.g., Parkinson's and Alzheimer's), cancer, cataracts and improving child growth [1–9]. Supplementation with coenzyme Q_{10} has been studied for its effect on hypertension [10,11], some neurodegenerative diseases [12], in reducing migraine headaches [13], as a possible marker of oxidative stress [14] and increased risk of atherosclerosis [15]. Direct measurement of the serum concentration of these important vitamins is essential for the evaluation of nutritional status of a patient. Due to the ease of simultaneous analysis vitamins A, E, β-carotene and coenzyme CoQ_{10} are often reported together as a profile of fat-soluble vitamins. Decreased concentrations of these principal molecules can reveal the need for increased dietary intake. The aim of this study was to create a robust, cost effective, clinically validated UHPLC method for simultaneous analysis of vitamin A, β-carotene, vitamin E and CoQ_{10} from human serum. The most common technique used for the separation of fat-soluble vitamins and CoQ_{10} has been reversed-phase high performance liquid chromatography (HPLC) [16–23]. Detection of fat-soluble vitamins and CoQ_{10} in HPLC is typically carried out using UV–vis [17–21] or fluorescence [19,22,23]. The recent introduction of ultra-high pressure liquid chromatography (UHPLC) offers the possibility of significantly increased efficiency of the chromatographic separation through the utilization of columns packed with smaller diameters particles (1.7 μm) that can withstand high

Abbreviations: CoQ_{10}, coenzyme Q-10; CVD, cardiovascular disease; GC, gas chromatography; LC, liquid chromatography; UV, ultraviolet; UHPLC, ultra-high pressure liquid chromatography; PDA, photodiode array; BHT, butylated hydroxytoluene; THF, tetrahydrofuran; LOD, limit of detection; LOQ, limit of quantitation; FDA, food and drug administration; IUPAC, International union of pure and applied chemistry; CLSI, Clinical and Laboratory Standards Institute; BEH, bridged ethylene hybrid.

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backpressures compared to the conventional HPLC instrumentation.

The present work describes a validated 2 min UHPLC method for analysis of four fat-soluble vitamins and CoQ_{10} that can be easily adopted for high-throughput clinical and pharmacokinetics studies.

2. Experimental

2.1. Chemicals and reagents

All-*trans*-retinol, α-tocopherol, β-tocopherol (50 mg/L in hexane), γ-tocopherol, β-carotene, CoQ_{10}, vitamin K_1, butylated hydroxytoluene (BHT) and sodium chloride were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile, ethanol, methanol, 2-propanol and hexane (95% n-hexane) were purchased from VWR International (Batavia, IL, USA). HPLC grade tetrahydrofuran (THF) was purchased from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Calibration and internal standard solutions

A stock calibration solution was prepared from commercial vitamins in BHT/THF solution (30 mg/L). The final concentration of the vitamins in the stock solution was as follows: retinol (200 mg/L), γ-tocopherol (200 mg/L), α-tocopherol (2000 mg/L), β-carotene (100 mg/L) and CoQ_{10} (300 mg/L). Five working standard solutions were prepared from the stock solution for the calibration of the assay. The highest working calibration standard solution (cal 5) was prepared by dilution of the stock calibration solution (0.5 mL) with ethanol (9.5 mL). The remaining four standard solutions were prepared by dilution of cal 5 with ethanol to the desired calibration ranges. The working calibration ranges for retinol, γ-tocopherol, α-tocopherol, β-carotene and CoQ_{10} were as follows: 0.18–10 mg/L, 0.18–10 mg/L, 1.80–100 mg/L, 0.09–5 mg/L and 0.27–15 mg/L, respectively. A stock internal standard solution (7600 mg/L) was prepared from the commercial vitamin K_1 in THF. The working internal standard solution (106.4 mg/L, 236 μM) was prepared in ethanol daily by addition of the vitamin K_1 stock solution (0.35 mL) and a BHT/THF solution (2.5 mL, 30 mg/L) to a 25 mL volumetric flask. All solutions were protected from light and stored in glass vials with Teflon-lined caps and stored at −70 °C. No solution was used for longer than 2 months.

2.3. Blood and QC samples

Serum collected from 12 h fasting patients was used for method development and was obtained from anonymous intra-laboratory patient samples with unknown medical histories. In addition blood drawn from fasting in-house volunteers with known medical histories was simultaneously collected in plasma (EDTA) and sera (SST red top) tubes and used for verification of the reference intervals. Protected from light the specimens were immediately separated by centrifugation and stored in amber plastic tubes at −20 °C until analysis (0 days to 2 months). The serum intra-laboratory samples were received frozen in amber plastic tubes and stored at −20 °C until analysis.

Two levels of quality control samples (normal and elevated) were prepared from freshly pooled sera, collected from in-house volunteers. Half of the serum was divided into micro-centrifuge tubes and frozen at −70 °C to be used as a normal control. A known amount of standard solution (1%, v/v) was added to the other half of the pooled sera to prepare the elevated control such that the concentration of each analyte will be within the calibration range and above the observed normal patient range. The samples were divided into tubes and stored at −70 °C. One sample of each control level was prepared and analyzed in duplicate with every batch for quality control.

2.4. Sample preparation

All preparations were conducted in dim light in order to minimize the photodegradation of the analytes. Specimen, calibrators, controls and a blank (0.5 mL) were individually mixed with the working internal standard solution (0.1 mL). A BHT/THF solution (1 mL, 30 mg/L) was added to precipitate proteins and protect the vitamins from radical oxidation during the sample preparation. Ethanol (0.5 mL) was added to the patient and control serum samples to match solvent composition to the calibrators. Addition of 3.5 mL NaCl (4%) to the calibrators and the blank and 3.0 mL to the patients and controls was followed by liquid–liquid extraction with hexane (4 mL). After centrifugation for 10 min at 2000 rpm, the organic layer from each tube was transferred to a clean glass tube and evaporated to dryness at 38 °C under nitrogen. The resulting oils were reconstituted with 2-propanol (0.15 mL), mixed well, transferred to an amber sample vial and injected into the UHPLC.

2.5. Instrumentation and chromatography

The chromatographic system consisted of a Waters ACQUITY UPLC system (Milford, MA, USA) with a PDA detector. Instrument control was accomplished by Waters MassLynx V4.1. Quantitation was performed by QuanLynx V4.1 at a wavelength of 324 nm for vitamin A, 291 nm for α- and 298 nm for γ- and β-tocopherol, 453 nm for β-carotene, 274 nm for CoQ_{10} and 266 nm for vitamin K_1. The results were calculated based on the response (area/internal standard area) of the analytes. The separation (2 μL injection volume) was carried out on an ACQUITY Shield RP_{18} BEH column (2.1 mm I.D. × 50 mm length, 1.7 μm particle size; Waters, Milford, MA, USA) maintained at 40 °C. The mobile phases used for the assay consisted of acetonitrile:de-ionized water (90:10, mobile phase A, MPA) and methanol:2-propanol (70:30, mobile phase B, MPB) delivered at a flow rate of 1.25 mL/min. Initial mobile phase conditions of 90:10 – MPA:MPB were held for 0.4 min, followed by introduction of MPB by a concave gradient (Waters # 8) from 0.4 to 1 min to a final composition of 20:80 – MPA:MPB. This composition was held for 0.4 min. At 1.4 min the system was returned to initial conditions: 90:10 – MPA:MPB and maintained for a 0.6 min post-run. The total run time was 2 min. A typical chromatogram of a standard and a patient, represented as the sum of all wavelengths between 260 and 500 nm is shown in Fig. 1.

2.6. Method validation

The method was validated including an evaluation of matrix effects, linearity, limit of detection (LOD) and quantitation (LOQ), accuracy, precision and stability following protocols from FDA, IUPAC and CLSI [24–26]. Matrix effects were evaluated using a statistical comparison of the slope of a calibration curve (area under the curve vs. standard concentration) obtained from four sets of standard solutions prepared with and without matrix present [27,28]. The Student's *t*-test was used to evaluate any difference or bias between the two slopes. A significant difference between the slopes or between each concentration is indicative of a matrix effect. To evaluate the ability of an internal standard to correct for any matrix effect, the calculation was repeated based on slope comparisons generated from calculated analyte response vs. standard concentration. The LOD and LOQ were determined from the average of five replicate calibration standard solutions

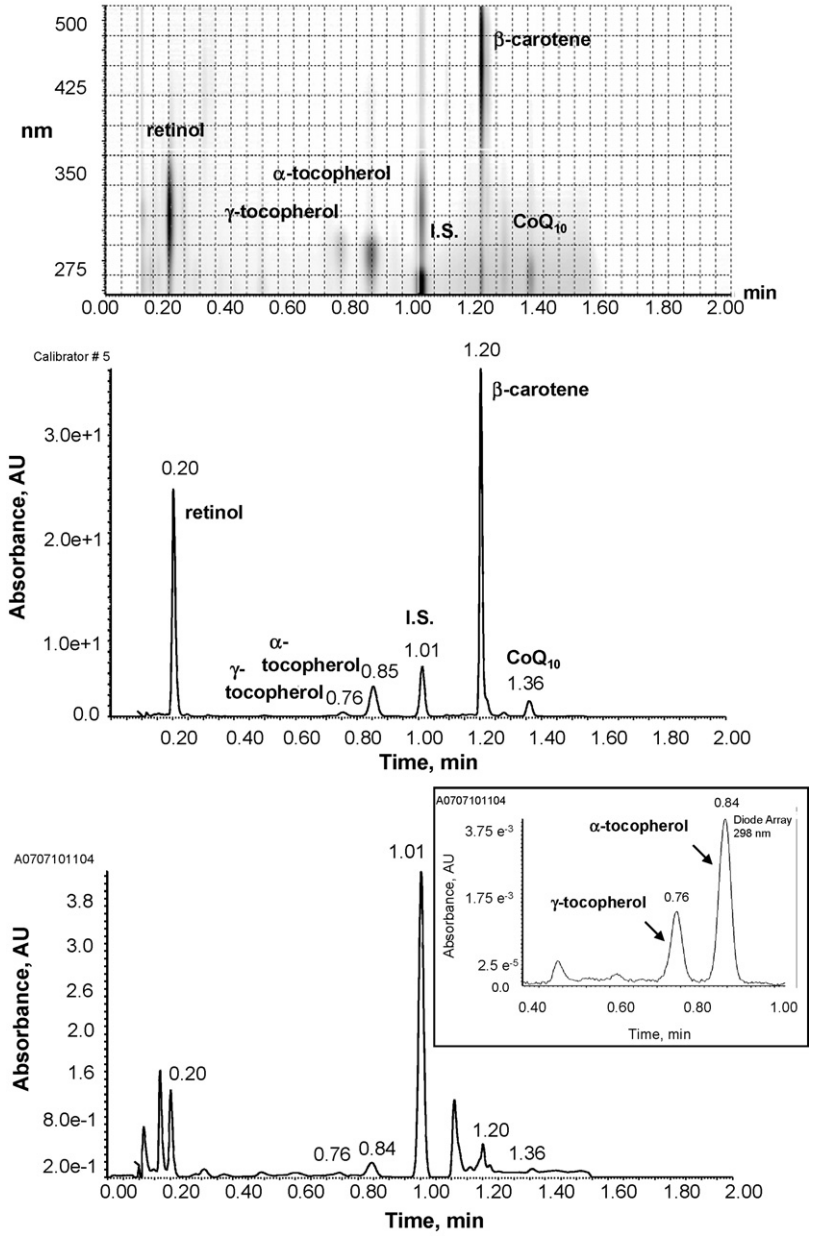

Fig. 1. UV map and chromatograms of five fat-soluble vitamins in working standard solution (upper and middle) and in human serum (bottom). The chromatogram in the small window above the bottom chromatogram shows the separation between γ- and α-tocopherol at 298 nm. The measured concentrations of the analytes in serum (on the bottom patient chromatogram) were as follows: retinol – 0.61 mg/L, γ-tocopherol – 1.38 mg/L, α-tocopherol – 5.50 mg/L, β-carotene – 0.10 mg/L and CoQ_{10} – 0.76 mg/L.

and calculated using the equations: LOD = 3 × (standard error for the y estimate)/slope. LOQ = 10 × (standard error for the y estimate)/slope. Accuracy was evaluated using a spike and recovery experiment from a pooled serum sample, spiked with a standard solution at five different concentration levels. The serum samples were spiked with no more than 10% of initial serum volume and the recovery was calculated based on the average of three successive measurements for each level and expressed as the percent of the analyte recovered. Inter- and intra-assay precision was determined using normal and elevated control samples. The inter-assay precision was calculated from two runs of each control level collected over 10 days ($n = 20$). The intra-assay precision was calculated from 10 runs of each control level collected in 1 day ($n = 10$). Stability was evaluated in prepared and unprepared samples stored under varying conditions to mimic shipping and storage. The long-term sample stability was evaluated on a freshly collected pooled serum sample at three different concentration levels, prepared by spiking the pooled sera with standard solutions, as well as an un-spiked blank. The resulting four levels were stored at three different conditions: room temperature, refrigerator (4 °C) and freezer (−20 °C). Additionally the stability was monitored throughout three freeze–thaw cycles. The stability was determined based on the % difference in the concentration from the initial reading. The preparation stability was evaluated from the comparison of quantitative results of three prepared samples evaluated over 3 days stored on the instrument. Patient ranges were collected from intra-laboratory samples. The values for normal and abnormal results were established using the MedCalc statistical program (MedCalc Software, Mariakerke, Belgium).

3. Results and discussion

3.1. Chromatography

A reversed-phase chromatographic method for the analysis of fat-soluble vitamins was developed using UHPLC separation with PDA UV–vis detection (260–500 nm). Three different ACQUITY UPLC columns were studied for chromatographic separation: phenyl, octadecyl (C_{18}) BEH and Shield RP_{18} BEH Shield. The phenyl column did not yield acceptable retention. The C_{18} and Shielded RP_{18} columns allowed comparable separation of the analytes. The RP_{18} BEH Shield column was selected for the assay, because it furnished slightly faster separation compared to the C_{18} BEH column (2 min – RP_{18} Shield compared to 2.5 min run time – C_{18} BEH). When combined with the UHPLC the BEH technology allowed for an excellent separation at a flow rate of 1.25 mL/min and pressure of ~600 bar (at 40 °C).

For the separation of γ- from α-tocopherol, which differ structurally by one methyl group, different solvent mixtures were tested by varying the amounts of de-ionized water in MPA and 2-propanol in MPB. The solvent mixtures allowing the best baseline separation of the two tocopherols were established followed by optimizing the ratios of the two eluents (MBA and MPB). Combination of 20:80 – MPA:MPB resulted in complete baseline separation of the two homologues, as seen in Fig. 1 on the UV map and the expanded inlay chromatogram (above the bottom chromatogram) at 298 nm (specific absorbance maximum for γ-tocopherol). In addition, separation of the isomeric vitamin E tocopherols (β-, γ- and δ-) has proven difficult by reversed-phase chromatography, as they differ only in the position of the methyl group on the aromatic ring [16,19,21,22]. Standard solutions of β- and γ-tocopherol were analyzed revealing only partial separation (β- 0.69 min, and γ- 0.71 min retention time) of the two positional isomers. However, the lack of full baseline separation was not a significant issue as the concentration of β-tocopherol in human serum has been reported to be very low when compared to that of α- and γ-tocopherol. Therefore, the two isomers are commonly reported together as the sum of γ- and β-tocopherol [19,22].

The fat-soluble vitamin K_1 was chosen as internal standard, because of its close structural resemblance with the analytes from the assay and its suitable retention time (elutes in the middle of the run, Fig. 1). In humans vitamin K_1 is present in low concentration levels (ng/mL) [29]. Patient samples prepared without addition of vitamin K_1 internal standard revealed no peaks in the area of elution of vitamin K_1, therefore, it could successfully be used as an internal standard for the method. As an added measure, the concentration of vitamin K_1 was prepared such that the final vial composition would be at a concentration 236 μM, which is nearly 5 orders of magnitude higher than the reported upper values of the normal population range (less than 3 nM) [30,31], therefore any vitamin K_1 variations from the patient sample will have very insignificant affect on the proper quantitation of the assayed fat-soluble vitamins, even in patients eating vitamin K enriched foods.

A simple sample preparation was used for the assay. The liquid–liquid extraction step utilized an aqueous NaCl solution, instead of the most commonly used water, allowing better separation of the organic from the aqueous layer while reducing emulsion formation. 2-Propanol was chosen for reconstitution over THF and EtOH/ACN mixture, because it furnished the best analyte solubility.

A mobile phase composition of de-ionized water (10%, v/v) in acetonitrile (90%, v/v) for MPA and 2-propanol (30%, v/v) in methanol for MPB were found to produce a stable baseline throughout the gradient while giving good compound resolution, particularity between γ- and α-tocopherol as highlighted by the inlay chromatogram in Fig. 1.

The reproducibility of the method was evaluated using both calibration and patient samples and the retention time reproducibility was calculated as a % difference from the initial injection. After 30 consecutive injections of a standard solution the change in retention times was less than 2.0% for each analyte (data not shown). The peak area reproducibility was evaluated as well by injecting three sets of six calibrators. The calculated % difference among the three injections for each level and analyte was less than 10% (data not shown).

In addition, the reproducibility of analyte retention time in over 70 patient samples within one batch was determined. The relative retention time was acceptably within ±0.02 min (data not shown).

3.2. Linearity and limits of detection and quantitation

A calibration curve consisting of a blank and six standard solutions was prepared. The linear range was as follows: 0.09–10.0 mg/L for retinol and γ-tocopherol, 0.93–100 mg/L for α-tocopherol, 0.05–5 mg/L for β-carotene and 0.14–15 mg/L for CoQ_{10}. These calibration ranges cover the 95% reference interval for all analytes. All slopes had a calculated correlation coefficient (r^2) greater than 0.999. The relative standard deviation (%R.S.D.) from the expected values was used to evaluate linearity over the calibration range. Calculated deviations from standard values, based on recovery, were less than 7% in all cases. The LOD and LOQ are listed in Table 1. Both LOQ and LOD indicate sufficient sensitivity for the method, given that clinically significant results are higher than the LOQ.

3.3. Accuracy

The accuracy of each analyte was evaluated based on the recovery of five concentration levels spiked into pooled serum (see Section 2.6 for more experimental details). As shown in Table 2, the averaged percent deviation from the theoretical value for the recovered spike for each analyte was less than the acceptable 15% [25].

3.4. Matrix effect study

A stock solution was prepared from commercial vitamins and CoQ_{10} in BTH/THF solution (30 mg/L). The final concentrations

Table 1
Sensitivity and population ranges of fat-soluble vitamins and CoQ_{10} from serum.

Analyte	Sensitivity		Patient ranges	
	LOQ (mg/L)	LOD (mg/L)	Adults (mg/L)[a]	Children (mg/L)[a]
Retinol	0.024	0.007	0.30–1.44	0.21–0.74
β + γ-Tocopherol	0.060	0.018	0.07–3.36	0.02–2.14
α-Tocopherol	0.261	0.078	4.24–30.01	3.80–23.27
β-Carotene	0.012	0.004	0.10–2.71	0.03–1.93
CoQ_{10}	0.028	0.008	0.48–3.01	0.28–4.28

[a] The 95% reference interval upper and lower limits of the range.

Table 2
Recovery of fat-soluble vitamins and CoQ_{10} from human serum.

	Retinol[a]	γ-Tocopherol[a]	α-Tocopherol[a]	β-Carotene[a]	CoQ_{10}[a]
Base					
Conc. (mg/L)	0.72	0.97	12.60	0.51	0.79
Spike (mg/L)	0	0	0	0	0
% Recovery	N/A	N/A	N/A	N/A	N/A
sp. 1					
Conc. (mg/L)	1.03	1.29	15.79	0.67	1.30
Spike (mg/L)	0.30	0.30	3.00	0.15	0.45
% Recovery	104	108	106	109	113
sp. 2					
Conc. (mg/L)	1.34	1.55	18.49	0.82	1.82
Spike (mg/L)	0.60	0.60	6.00	0.30	0.90
% Recovery	104	97	98	104	114
sp. 3					
Conc. (mg/L)	2.00	2.07	23.76	1.06	2.78
Spike (mg/L)	1.20	1.20	12.00	0.60	1.80
% Recovery	107	92	93	92	110
sp. 4					
Conc. (mg/L)	3.45	3.52	38.33	1.73	4.87
Spike (mg/L)	2.40	2.40	24.00	1.20	3.60
% Recovery	114	107	107	102	113
sp. 5					
Conc. (mg/L)	5.93	5.53	58.60	2.71	8.18
Spike (mg/L)	4.80	4.80	48.00	2.40	7.20
% Recovery	109	95	96	92	103

[a] The concentration values were based on the mean of three samples analyzed in the same batch.

of the analytes were as follows: retinol (200 mg/L), γ-tocopherol (200 mg/L), α-tocopherol (2000 mg/L), β-carotene (100 mg/L) and CoQ_{10} (300 mg/L). Calibration standard # 5 was prepared from the stock solution (0.5 mL) and pooled serum (9.5 mL, for the matrix curve) or ethanol (9.5 mL, for the non-matrix curve). The other four calibration solutions (500 μL final volume) were prepared by dilution of cal 5 with serum pool or ethanol to the desired concentration ranges (Part 2.2). Four sets of matrix and non-matrix calibrators (cal 1–5) and a blank were prepared and run as patients according to the procedures outlined in Section 2.4. Two linear regression plots were generated for all analytes. Plots for the area under the curve (AUC) and the response factor vs. the standard concentrations were compared. All of the analytes had a *t*-value less than 2.45, which indicated that the two slopes are considered statistically the same and therefore the matrix does not produce a significant effect on the area or response of the analytes or internal standard. A representative of the regression plots is shown in Fig. 2 for α-tocopherol.

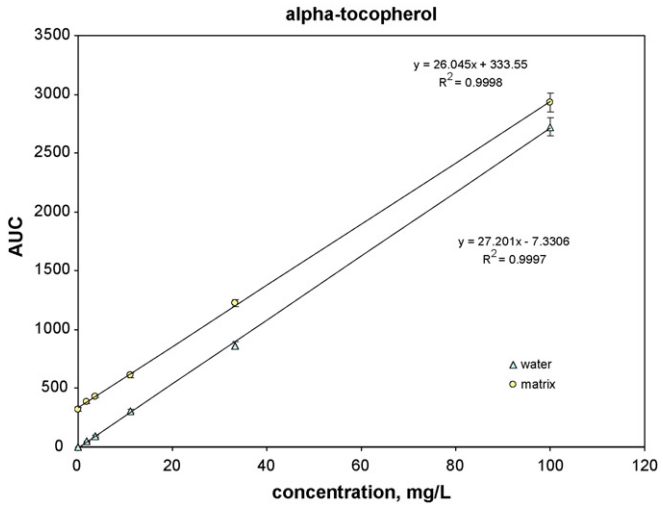

Fig. 2. The linear regression plots of AUC vs. concentration of calibration solution in water and serum matrix, used to determine the matrix effects for α-tocopherol.

3.5. Reference interval

The reference ranges were established on multiple 50 mm RP_{18} Shield BEH UHPLC analytical columns from intra-laboratory samples of 160 fasting adults (ages 13 and up) and 120 fasting children (0–12 years) with unknown medical histories. In addition, the adult normal ranges were confirmed with samples from 20 healthy in-house volunteers. The upper and lower limits were calculated using a 95% reference interval (Table 1).

The calculated LOD and LOQ for all analytes were below the low limit of the normal range for adults. The calculated LOD value for γ-tocopherol (0.018 mg/L) was close to the lower limit of the normal range for children, therefore concentration values for this vitamin E analog, which were lower than its calculated LOQ (0.06 mg/L) were reported as less than the LOQ.

3.6. Precision

Inter-assay precision was measured by the variation of two control concentrations (normal and elevated) over 10 days. The intra-assay precision was calculated from 10 samples of each control level measured in a batch. The % C.V. for all analytes from intra- and inter-assay precision studies was below 5% and 10% for each level, respectively (Table 3).

3.7. Stability

A light protected pooled serum sample (500 μL) and three spiked serum samples from each storage condition: room temperature (25 °C), refrigerator (2–4 °C) and freezer (−20 °C) were prepared and analyzed over several days. The results from the study show that the analytes were stable after 2 days of storage at room

Table 3
Inter- and intra-assay precision (% CV) of fat-soluble vitamins calculated on two levels of serum quality controls.

Control level	Retinol[a] (mg/L) (%CV)[b]	γ + β-Tocopherol[a] (mg/L) (%CV)[b]	α-Tocopherol[a] (mg/L) (%CV)[b]	β-Carotene[a] (mg/L) (%CV)[b]	CoQ_{10}[a] (mg/L) (%CV)[b]
Normal					
Intra (n = 10)	0.69 (2.1)	0.96 (3.6)	13.29 (2.3)	0.49 (2.6)	1.04 (4.6)
Inter (n = 20)	0.72 (6.1)	0.89 (8.3)	13.51 (6.7)	0.53 (7.3)	1.07 (8.2)
Elevated					
Intra (n = 10)	2.63 (2.7)	7.56 (2.8)	2.17 (2.6)	3.02 (3.1)	5.61 (2.4)
Inter (n = 20)	2.67 (3.2)	7.11 (7.5)	53.76 (3.9)	3.15 (4.9)	5.52 (7.5)

[a] The average concentration from 10 experiments (within run) and 20 experiments (between run).
[b] % CV, coefficient of variation (or %R.S.D., relative standard deviation).

temperature. All analytes were stable for three freeze–thaw cycles, for 3 weeks of refrigerator and for 8 weeks of freezer storage.

To test the stability of a prepared sample, three different patient samples were freshly prepared and analyzed in duplicate each day over a 4-day period. Between readings, the samples were protected from light and stored in the instrument's refrigerated storage compartment at 10 °C. The % difference of analyte concentration from the initial day of the preparation to the day of analysis was calculated for each analyte. No analyte deviated more than 15% from the initial values over a 3-day period.

4. Conclusion

In summary, we are presenting the first UHPLC method for the analysis of four fat-soluble vitamins and CoQ_{10} from human serum. The selected chromatographic conditions allowed complete baseline separation of the two vitamin E homologues (α- and γ-tocopherol) and therefore their accurate quantitation. The total run time of 2 min is two to three times faster compared to currently reported HPLC methods for analysis of similar panel of nutrients [17,18,21,22]. The assay has been operated for approximately 1 year in our lab and no interferences have been observed.

The method was validated by linearity, accuracy, matrix effect studies, LOD, LOQ, precision and stability. Patient ranges were established for children and adults based on more than 280 serum samples. The established LOQ values for all analytes were suitable for clinical evaluation.

The sensitivity in addition to the low cost (from the classical liquid–liquid extraction sample prep, relatively cheap standards and instrumentation used for the analysis) of the assay allow for a fast evaluation of the nutritional status of patients of various age groups.

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