Al Ameen J Med Sci 2018;11(3):147-153 ● US National Library of Medicine enlisted journal ● ISSN 0974-1143

ORIGINAL ARTICLE CODEN: AAJMBG

Concurrent analysis of fat and water soluble vitamins in biological fluids using reverse phase-high performance liquid chromatography technique

Annu Vats, Gurseen Rakhra, Daisy Masih and Som Nath Singh*

Nutrition Division, Defence Institute of Physiology and Allied Sciences, Lucknow Road, Timarpur, Delhi-110054 India

Abstract: *Background:* Vitamins are essential micronutrient which plays an important role as cofactor of enzymes in energy metabolism. Methods used for vitamin analysis in biological samples are usually based on chemical or microbiological assays that are not very accurate and is time-taking. HPLC method were developed and validated for the simultaneous detection and quantitation of three fat soluble and eight water-soluble vitamins in plasma and urine samples. *Methods:* Separation was achieved at 40˚C on a reversed-phase C18 column using isocratic mode with methanol and ethanol (75:25) for fat soluble and multi-step gradient mode with methanol and water for water soluble vitamins. Total run time was 20 minutes for fat soluble and 34 minutes for water soluble vitamins. Detection was performed with quaternary diode array detector set at maximum absorption wavelengths for quantification of each vitamin. Spectral comparison was used for peak identification in real plasma samples. *Results:* The novel analytical method detailed in this paper has proved to be specific, robust, and time-efficient for the simultaneous detection and quantification of fat and water-soluble vitamins in complex biological matrices such as plasma and urine. *Conclusion:* Method reported can be used in multivitamin analysis in matrixes where low concentrations of vitamins are expected. Recovery percentages ranged from 93% to 100%.

Keywords: Isocratic mode, multi-step gradient mode, quaternary diode array detector, Run time, time-efficient method.

Introduction

The term "vitamin" is used to describe certain organic compounds that are needed by the body but that cannot be synthesized by the body. They mainly serve as catalysts for certain reactions in the body. The amounts of vitamins required are very small, perhaps hundredths of grams. The main source of vitamins is our diet [1]. Vitamins are classified on the basis of their solubility i.e. fat-soluble (lipid soluble) and water-soluble. Vitamins A, D, E and K are fat-soluble and stored in the body, whereas Vitamin C and B are water soluble and excess of which are flushed out by kidneys. Vitamin B-complex group contains eight water soluble vitamins viz. thiamin (vitamin B1), riboflavin (vitamin B2), niacin (vitamin B3), vitamin B6 (pyridoxine), folate (folic acid), vitamin B12, biotin and pantothenic acid [1]. Except the above said vitamins the body also needs vitamin C, also known as ascorbic acid. Benefits of vitamin C include protection against immune system deficiencies, eye disease, prenatal health problems, cardiovascular problems, collagen synthesis. Due to the solubility; fat-soluble vitamins (A, D, E, and K) gets stored in the body fat reserves of adipose tissue for long periods of time and if consumed in excess they poses a risk for toxicity. Eating a normal, well-balanced diet will not lead to toxicity in otherwise healthy individuals [1].

Various methods for vitamin analysis are based on time-consuming microbiological assay that also lacks specificity [2]. In addition, vitamin extraction involves pretreatment through complex chemical reactions followed by individual methods for the determination of each vitamin. Now-a-days, interest has been increasing in instantaneous determination of vitamins using a time efficient methodology. Thus, various analytical methods have been developed over recent years [3]. A vast majority of studies focused on multivitamin analysis in food matrices, drinks, polyvitaminated premixes,

© 2018. Al Ameen Charitable Fund Trust, Bangalore

and vitamin supplements [4]. On the other hand, limited number of validation studies has been conducted for simultaneous analysis of multivitamins in biological samples (blood and urine) and with very few results in terms of lengthy sample preparation steps and method's robustness and reproducibility [5-7]. Due to the lack of a validated analytical test for concurrent analysis of multivitamins in biological samples, the aim of this study was to develop and validate two novel HPLC based methods one for simultaneous detection and quantification of fat soluble vitamins and other for water soluble vitamins in biological fluids viz plasma and urine.

Material and Methods

Sample Collection: Fasting venous blood samples were collected during morning hours (0700-0800 h) in heparin tubes. Heparin tubes were centrifuged for 15 min at 1000 x g at room temperature to fractionate blood into upper plasma and lower RBC layer. Plasma samples were stored in two different vials, one containing 10µl of 1 M dithiothretiol (DTT) used for estimation of water soluble vitamins and plain plasma vials for estimation of fat soluble vitamins kept on -80°C until analysis. 24 hours urine samples were collected in 6N HCl containing jerry cans. Total urine volume was recorded and 10 ml sample was stored at -80˚C until analysis. Both fat and water soluble vitamins were estimated in plasma samples obtained from ten healthy individuals for proving applicability of the method developed.

Fat Soluble Vitamin Estimation: Estimation of fat soluble vitamins in plasma samples was done using method developed by Kandar et. al. 2012 [8] with modifications in terms of sample volume, hexane volume, chromatography column features and using vacuum concentrator instead of nitrogen evaporator.

Standard and Reagents: Fat soluble vitamins employed in this study (retinol, α-tocopherol, and cholecalciferol) and internal standard (retinyl acetate) were purchased from Sigma Aldrich and were of highest purity grade available (95%). HPLC grade ethanol, methanol and hexane were purchased from Merck. Trifluoroacetic acid (TFA) of protein chemistry grade (>99.5%) was purchased from Thermo Scientific, Fisher Scientific. Ultra-pure HPLC grade water (milliQ) was used throughout the entire protocol.

Chromatographic conditions: A Thermo fisher Scientific 3000 series UHPLC was used for the analysis and quantification of vitamins in plasma and urine samples. The chromeleon™ system controlled the whole chromatography system.

Vitamins were separated in reversed phase chromatography column (Thermo scientific Acclaim™ C18 120A column; Betasil, 4.6 x 100 mm, U.S.A) 5u Guard cartridge (4 x 10 mm, Cranbury, NJ, U.S.A) using an isocratic elution with a mobile phase i.e. methanol : ethanol in a ratio of 75:25 consisting of 0.01% TFA aqueous [8]. The flow rate was adjusted to 0.8 ml/min [8] for a total run time of 20 minutes. Injection volume was 10 µl. Column temperature was kept constant at 40˚C [8] for better separation. Quaternary diode array detector (DAD) was used for detection and quantification at the maximum absorption wavelength for each vitamin as follows: 325 nm for retinol, 292 nm for α-tocopherol and 280 nm for cholecalciferol.

Standard preparation: The standard stock solutions of fat soluble vitamins were prepared weekly by weighing retinol - 100 mg/L, α-tocopherol - 1000 mg/L, Vitamin D - 1000 mg/L in HPLC grade ethanol. The solution was transferred by pouring in amber-glass bottle for storage at 4˚C. The final concentration for each vitamin used in working standard solution was 10 times diluted from the stock solution, i.e. 10 mg/L for retinol and 100 mg/L for α-tocopherol and vitamin D.

Sample Preparation: Experiments were carried out to identify a sample preparation procedure that would allow simultaneous detection of fat soluble vitamins in plasma samples. The methodology involves liquid-liquid phase extraction was carried out by transferring to a glass analysis tube 400 µl of fresh or freshly thawed plasma. 400 µl of ethanol was added and the tubes were briefly vortex mixed. The addition of 1 ml n-hexane was done to extract lipid soluble matrix

© 2018. Al Ameen Charitable Fund Trust, Bangalore

components mainly fat soluble vitamins. Solutions were vortex mixed for 5 minutes. Tubes were then centrifuged at 10000 rpm for 5 minutes at 4˚C. The upper layer, i.e. hexane organic phase was collected and placed in a new capped micro centrifuge tube. Tubes were placed in a vacuum concentrator instrument (Thermo Scientific, Fisher Scientific, Loughborough, UK) to dry. When the supernatant from the organic phase was completely dried, samples were resuspended in HPLC vials containing 0.01% TFA in ethanol.

Water Soluble Vitamin Estimation: Estimation of water soluble vitamins in plasma samples was done using method developed by Giorgi et. al. 2012 [9], with modifications in terms of sample collection, gradient programming for solvent flow, chromatography column features.

Standard and Reagents: Water soluble vitamins employed in this study (Thiamine (B1), pyridoxal phosphate (PLP) (B6), Ascorbic acid (C), Niacin (B3), Pantothenic acid (B5), Folic acid (B9), Riboflavin (B2), cobalamin (B12)) were purchased from Sigma Aldrich and were of highest purity grade available (95%). As described earlier all solvents used were of HPLC grade.

Chromatographic conditions: Vitamins were separated in reversed phase chromatography column (Thermo scientific Acclaim^{TM} C18 120A column) using a multistep gradient elution with a mobile phase consisting of methanol (Solvent A) and 0.01% TFA aqueous solution in water (solvent B). Linear gradient profile (A : B) started at 5 : 95 and it was kept constant for 2 minutes, then linearly increased up to 15 : 85 during the next 3 minutes. Again linear increase in gradient profile was achieved upto 22 : 78 for the next 5 minutes and then linearly increased up to 60 : 40 during the next 10 minutes.

Again a linear increase in gradient profile was achieved during the next 8 minutes and it was kept constant 2 minutes and finally linearly decreased up to 5 : 95 during the last 4 minutes of run time. The flow rate was adjusted to 0.5 ml/min [9] for a total run time of 34 minutes. Injection volume was 10 µl. Column temperature was kept constant at 40˚C [9] for better separation. Maximum absorption wavelengths for each vitamin are as follows: 257nm (B1), 210nm (B6), 257nm (C), 210nm (B3), 204nm (B5), 280nm (B9), 257nm (B2), 260nm (B12). But we used quaternary diode array detector (DAD) for detection and following four absorption wavelengths were selected so that each vitamins can be detected: 280 nm, 257 nm, 210 nm, 204 nm.

Standard preparation: The standard stock solutions of fat soluble vitamins were prepared weekly by weighing each standard, i.e. B1, B6, C at a concentration of 50 ng/µl and B2, B3, B5, B9 and B12 at a concentration of 100 ng/µl and dissolved in water. The solution was transferred by pouring in amber-glass bottle for storage at 4˚C. The final concentration for each vitamin used in working standard solution was 10 times diluted from the stock solution.

Sample Preparation: A methodology that helps in simultaneous extraction of all eight water soluble vitamins from plasma samples using liquid-liquid phase extraction was carried out by transferring to a glass analysis tube 400 µl of fresh or freshly thawed plasma containing DTT. The addition of 1 ml n-hexane was done to extract lipid soluble matrix components mainly fat soluble vitamins. Solutions were vortex mixed for 5 minutes. 400 µl of ethanol was added and the tubes were briefly vortex mixed. Tubes were then centrifuged at 10,000 rpm for 5 minutes at 4˚C. The lower layer, i.e. aqueous phase was collected and placed in a new capped micro-centrifuge tube. Tubes were placed in a vacuum concentrator instrument (Thermo Scientific, Fisher Scientific, Loughborough, UK) to dry. When the aqueous extract was completely dried, samples were resuspended in HPLC vials containing 0.01% TFA in water before injection in HPLC.

Method Development:

System Suitability: The system suitability was evaluated by five replicate analysis of a standard ethanolic mixture of fat soluble vitamins and standard aqueous mixture of water-soluble vitamins. The percent coefficient of variation (%CV) of the peak area and the retention time of both fat and water-soluble vitamins were accepted up to the limit of $\pm$ 5%.

© 2018. Al Ameen Charitable Fund Trust, Bangalore

Linearity (Calibration Curve): For linearity test, five standard mixtures of both fat and water soluble vitamins were run and calibration curves were constructed at final on-column concentrations of 1.25, 2.5, 5.0, 7.5, 10.0 mg L^{-1}. The internal standard, retinyl acetate (for fat soluble vitamins) and theobromine (for water soluble vitamins), was kept at a constant concentration of 2 ng/μL.

Specificity: The ability of an analytical method to detect the analyte peak in presence of all the matrix components was checked by specificity testing. In this case, a standard mixture of both fat and water-soluble vitamins and the internal standard at known concentration (20 ng μL−1 and 2 ng μL−1, resp.) were spiked in a matrix of simulated plasma.

Limits of Detection and Quantitation (Sensitivity): Limit of detection (LOD) and limit of quantitation (LOQ) were estimated from the signal-to-noise ratio. LOD is defined as the lowest concentration of the analyte that can be detected and is three times the baseline noise. LOQ is defined as the lowest concentration that provides a signal-to-noise ratio higher than 10, with precision (%CV) and accuracy (%bias) within their acceptable range (10%).

Recovery Studies: Calculation of the percentage recovery rate (% recovery) was done using the experimental response values and values provided by the calibration curves for the same quantity of analyte. Student's t-test was performed to assess whether the recovery rate was significantly different from 100% at $P< 0.05$.

Statistical Analysis: Analysis of data collected in this study was done using graphPad Prism version-5 software by one way analysis of variance (ANOVA) and by independent samples Student's t-test. Linear regression analysis using least square method was used to test the model. Correlation coefficient was calculated and the results of the statistical analysis were considered significant if their corresponding P values were less than 0.05.

Results and Discussion

System Suitability: Results from system suitability studies are reported in Table 1.

The optimized HPLC method for determination of vitamins in biological matrices (column temperature = 40◦C; flow rate = 0.8mLmin^{-1} for Fat soluble vitamins and 0.5 mLmin^{-1} for Water soluble vitamins). Mean peak areas of 5 injections of aqueous standard solution containing 20 ngmL^{-1} of each water-soluble vitamin.

Table-1: System suitability test results for the optimized HPLC method

Vitamin	Retention Time (min) (%CV)	Capacity Factor (K')	Selectivity (α)	Resolution (Rs)	Tailing Factor	Plate Count	Mean Peak areas (mAU)
A	4.6 (0.9)	2.5	1.1	2.28	0.65	3265	4.44
E	6.6 (0.8)	3.2	1.05	2.46	0.81	3189	25.23
D	7.5 (0.7)	4.0	1.2	2.71	0.65	916	28.25
B1	3.4 (0.85)	1.2	1.22	3.01	079	2862	22.59
B6	4.7 (0.75)	2.5	1.35	2.6	0.71	2360	72.38
C	7.2 (0.76)	3.9	1.41	2.91	0.82	4047	3.25
B3	8.9 (0.85)	4.4	1.2	3.04	0.69	1389	8.5
B5	15.0 (0.95)	7.5	1.15	2.1	0.73	19022	12.31
B12	20.4 (1.22)	8.5	1.7	2.61	0.80	57644	132.39
B9	23.0 (1.6)	8.7	1.6	2.5	0.68	86041	33.76
B2	24.2 (1.8)	8.8	1.3	2.1	0.74	19393	36.58

© 2018. Al Ameen Charitable Fund Trust, Bangalore

Plasma Sample Extraction Method Development: A suitable sample preparation procedure was used that allows better extraction of three fat soluble (A, D, E) and eight water-soluble vitamins from plasma. Results are reported in Table 2.

The sample preparation method optimization for vitamins spiked in plasma samples processed using liquid-liquid extraction technique for both fat and water soluble vitamins. Results are reported as mean of peak area ± RSD (relative standard deviation) (n=3).

Table-2: Results from the sample preparation method

Vitamins	Peak Area (mAU*min)	% RSD
A	4.44	0.22
E	25.23	4.7
D	28.25	5.3
B1	22.59	5.1
B6	72.38	2.3
C	3.25	0.04
B3	8.5	0.41
B5	12.31	0.61
B12	132.39	4.5
B9	33.76	2.4
B2	36.58	3.0

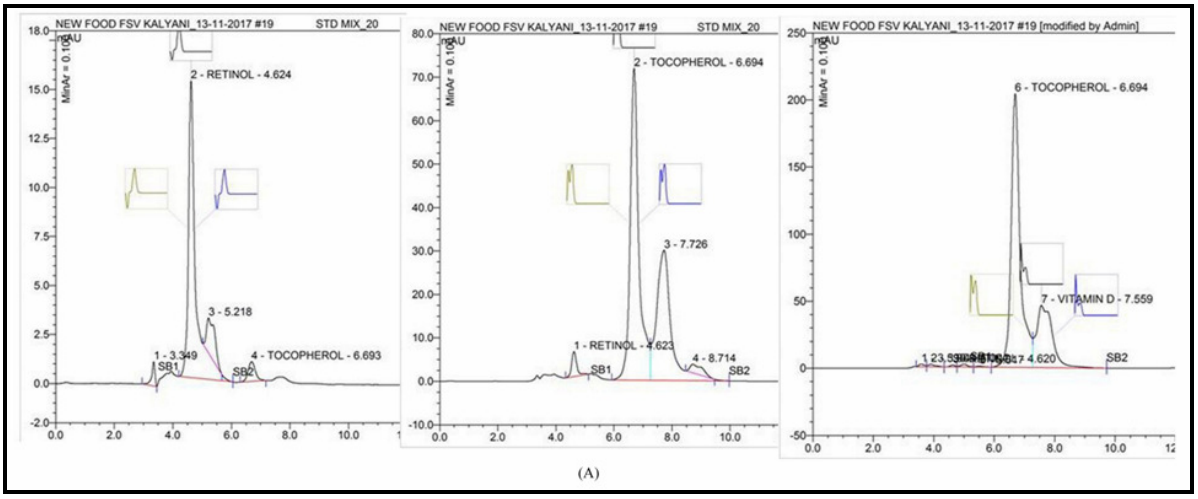
Method Validation:

Specificity and Linearity Studies: Representative chromatograms of standard are depicted in, respectively, Figures 1 (A) and 2 (A). Vitamins elute in a specific order and in groups depending upon their chemical properties and interaction with the analytical column. Figure 1(B) and 2(B) represents the chromatogram of real plasma sample.

As expected, retinol followed by tocopherol and then vitamin D gets eluted. Similarly in case of water soluble vitamins, polar vitamins (B1, B6, C, B3) elute first, followed by B5 and finally by low polar vitamins (B12, B9, B2). Retention times were as following: 4.6 ± 0.03 [A], 6.6 ± 0.05 [E], 7.5 ± 0.04 [D] for fat soluble and for water soluble 3.4 ± 0.03 [B1], 4.7 ± 0.04 [B6], 7.2 ± 0.06 [C], 8.9 ± 0.07 [B3], 15.0 ± 0.09 [B5], 20.4 ± 0.06 [B12], 23.0 ± 0.08 [B9], 24.2 ± 0.07 [B2].

As reported in Figure 1(A) and 2 (A); peaks are well resolved and symmetric. Peak identification and purity were investigated by comparing UV spectra of each individual vitamin when analyzed in mixtures and by running standard samples containing only one vitamin. When comparing standard chromatogram with chromatograms obtained from real plasma as represented in figure 1(B) and 2 (B), no major interference was noted from endogenous substances naturally present in human plasma. Two unidentified peaks were noted in spiked sample chromatogram; however, they did not interfere with vitamins quantitation.

Fig-1: Fat soluble vitamin chromatogram: (A)- Standard Chromatogram, (B)- Real Plasma Sample



© 2018. Al Ameen Charitable Fund Trust, Bangalore

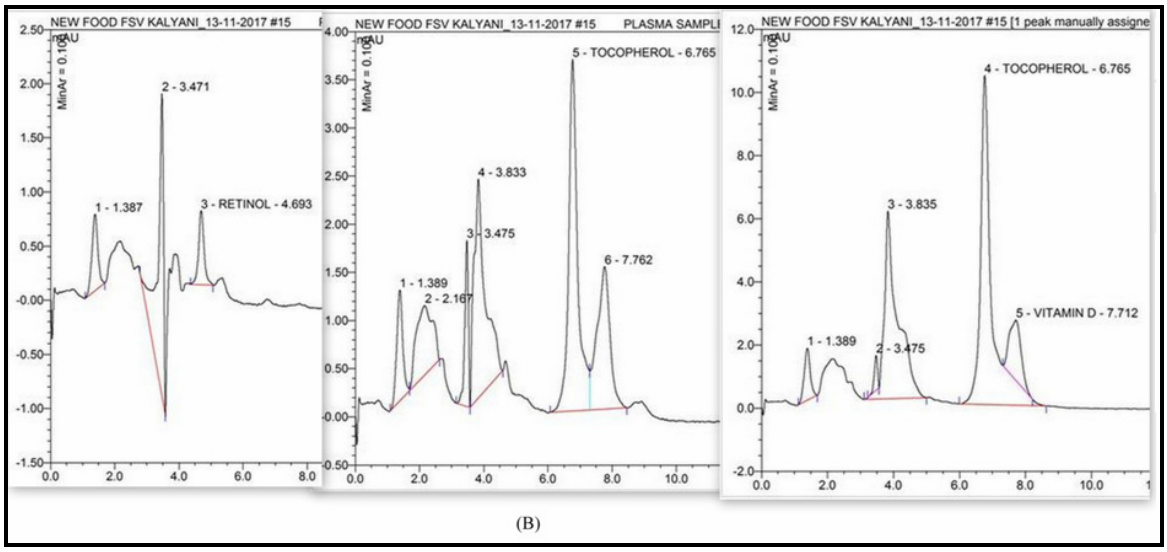
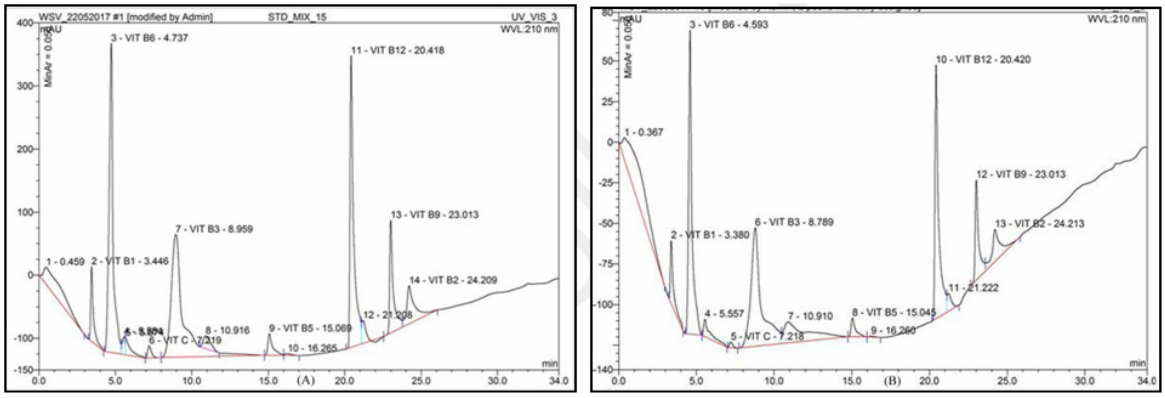


Fig-2: Water soluble vitamin chromatogram: (A)- Standard Chromatogram, (B)- Real Plasma Sample



Linearity was tested by running six standard mixtures of water-soluble vitamins at final, on-column concentrations of 1.25, 2.5, 5.0, 7.5, 10.0 mg L^{-1}. The method was linear across the whole range of concentrations (Table 3). For all peaks, there was a very tight relationship between the amount of vitamins and the detectors response as indicated by R^2 values that exceeded 0.996 (±0.002). Linearity of the method was also investigated in spiked plasma samples (data not shown).

No statistically significant difference was observed between slopes of regression lines generated by aqueous- and plasma-based standards (Student's t-test not significant, $P > 0.05$). So for quantitation purposes, aqueous-based standards were used to calibrate the instrument response when analyzing trial samples.

Table-3: Linear regression calibration curve representing regression coefficient and linear regression slope

Vitamin	Linear Regression Line Slope	Regression Coefficient (R^2)	Limits of Detection (ng μL^{-1})
A	0.38	0.989	0.5
E	0.72	0.998	1.0
D	0.29	0.997	1.0
B1	0.49	0.996	0.5
B6	1.48	0.998	0.5
C	0.1	0.999	0.5
B3	1.80	1	1.0
B5	0.12	0.979	1.0
B12	1.28	0.999	1.0
B9	0.94	0.998	1.0
B2	0.66	0.999	1.0

Calibration curves were constructed using six standard concentrations of eight water-soluble vitamins prepared in double distilled water, and they were run in triplicate. For each

© 2018. Al Ameen Charitable Fund Trust, Bangalore

curve, peak-areas of vitamins were plotted against the nominal (theoretical) vitamins concentration. Calibration curves were generated by weighted (1/y) linear regression analysis. Detection/ quantitation limits were determined by analysis of six standard solutions and three spiked plasma samples with final concentrations ranging from 1.25 to 10 mg L^{-1} each vitamin. Recovery tests were performed in triplicate by spiking blank plasma sample before deproteinisation and liquid-liquid extraction by 20 ng/µL of each vitamin. This concentration was selected as a compromise between the most possible plasma vitamins concentration to be found in real samples. Results were not significantly dissimilar to 100% [Student's t-test not significant, $P > 0.05$]. Recovery experiments were repeated at 75%, 50%, 25%, and 10% of the above vitamins concentrations. Recovery percentages ranged from 93% to 100% at all concentrations.

Vitamin concentration in plasma samples: Table 4 represents the vitamin concentration in plasma samples.

Table-4: Vitamin Concentration in plasma samples

Vitamin	Concentration
Vitamin A	95.53 ± 3.37 µg/dl
Vitamin E	11.16 ± 0.35 mg/L
Vitamin D	3.63 ± 0.22 µg/dl
Vitamin B1	5.04 ± 0.17 µg/dl
Vitamin B6	23.72 ± 1.24 µg/L
Vitamin C	6.88 ± 0.17mg/L
Vitamin B3	4.14 ± 0.22 µg/ml
Vitamin B5	90.91 ± 3.44 µg/L
Vitamin B12	245.4 ± 8.04 ng/L
Vitamin B9	12.73 ± 0.88 ng/ml
Vitamin B2	8.24 ± 0.63 µg/L

Conclusion

Methods described in this paper are reliable, specific, sensitive, and time-efficient for detection and quantification of fat and water soluble vitamins and can be used for routine clinical investigations involving analysis in biological samples, even with very low concentrations.

References

1. Gerald F Combs ed. The Vitamins: Fundamental Aspects in Nutrition and Health. *Academic Press: London.* 2017; 5:34-52.
2. Shabangi M and Sutton JA. Separation of thiamin and its phosphate esters by capillary zone electrophoresis and its application to the analysis of water-soluble vitamins. *Journal of Pharmaceutical and Biomedical Analysis,* 2005; 38(1):66-71.
3. Yin C, Cao Y, Ding S, and Wang Y. Rapid determination of water- and fat-soluble vitamins with microemulsion electrokinetic chromatography. *Journal of Chromatography A*, 2008; 1193(1-2):172-177.
4. Ghorbani AR, Momenbeik F, Khorasani JH and Amini MK. Simultaneousmicellar liquid chromatographic analysis of seven water-soluble vitamins: optimization using supermodified simplex. *Analytical and Bioanalytical Chemistry,* 2004; 379(3):439-444.
5. Chatzimichalakis PF, Samanidou VF, Verpoorte R and Papadoyannis IN. Development of a validated HPLC method for the determination of B-complex vitamins in pharmaceuticals and biological fluids after solid phase extraction. *Journal of Separation Science,* 2004; 27(14):1181-1188.
6. Gentili A, Caretti F, D'Ascenzo G et al. Simultaneous determination of water-soluble vitamins in selected food matrices by liquid chromatography/electrospray ionization tandem mass spectrometry. *Rapid Communications in Mass Spectrometry,* 2008; 22(13):2029-2043.
7. Klejdus B, Petrlov´a J, Pot˘e˘sil D et al. Simultaneous determination of water- and fat-soluble vitamins in pharmaceutical preparations by high-performance liquid chromatography coupled with diode array detection. *Analytica Chimica Acta,* 2004; 520(1-2):57-67.
8. Kandar R, Novotna P and Drabkova P. Determination of Retinol, α-Tocopherol, Lycopene, and β-Carotene in Human Plasma Using HPLC with UV-Vis Detection: Application to a Clinical Study. *Journal of Chemistry.* 2012; 2013:7.
9. Giorgi MG, Howland K, Martin C, Bonner AB. A Novel HPLC Method for the Concurrent Analysis and Quantitation of Seven Water-Soluble Vitamins in Biological Fluids (Plasma and Urine): A Validation Study and Application. *Scientific World Journal.* 2012; 8. Article ID 359721.

Cite this article as: Vats A, Rakhra G, Masih D and Singh SN. Concurrent analysis of fat and water soluble vitamins in biological fluids using reverse phase-high performance liquid chromatography technique. *Al Ameen J Med Sci* 2018; 11(3):147-153.

*All correspondences to: Dr. Som Nath Singh, Nutrition Division, Defence Institute of Physiology and Allied Sciences, Lucknow Road, Timarpur, Delhi-110054 India. E-mail: nutrition@dipas.drdo.in

© 2018. Al Ameen Charitable Fund Trust, Bangalore