Future Journal of Pharmaceutical Sciences

RESEARCH Open Access

Chromatographic method for estimation of vitamin E from dried blood spot sample

Archana Mohit Navale[1*], Vichika R. Patel[2] and Falguni B. Tandel[2]

Abstract

Background: We developed a selective bioanalytical RP-HPLC method for estimation of vitamin E from dried blood spot (DBS) sample, a potential technique which can be used for population-based epidemiological studies. Vitamin E was extracted from DBS by using liquid-liquid extraction technique with methanol (100% v/v) as reconstituting solvent for the residue. Alpha tocopheryl acetate was used as internal standard. Samples were analyzed directly on HPLC with C_{18} (250 × 4.6 mm × 5 μm) Phenomenex column. The mobile phase used was methanol to water (99:1% v/v) at a flow rate of 1.4 mL/min. The detector wavelength used was 292 nm.

Results: The retention time observed for vitamin E and internal standard was 10.225 ± 0.00075 min and 13.580 ± 0.00075 min respectively. The vitamin E calibration curve was found to be linear over the range of 0.625 to 60 μg/mL. The limit of quantification for vitamin E was found to be 0.1 μg/mL. Accuracy of the developed method was found to be 103.179%, 101.625%, and 100.174% with percentage of coefficient of variation of 0.0161, 0.0215, and 0.2790 for HQC, MQC, and LQC samples respectively which were within USFDA acceptance limit of ± 15 to ± 20%.The intraday and interday precision expressed as coefficient of variation were 0.0191–0.0841% and 0.0074–0.0252% respectively.

Conclusions: The method represents a simple, rapid, specific, accurate, and precise method for estimation of vitamin E in human blood using DBS technique. The developed method can be further evaluated with respect to effect of matrix variability before it can be used in clinical setting.

Keywords: Vitamin E, RP-HPLC, Dry blood spot (DBS), Alpha tocopheryl acetate

Background

Vitamin E is an important lipid phase antioxidant. Oxidative stress in lipid component of cell leads to significant disturbance of cell homeostasis. Such stress has been identified as contributory factor in pathogenesis of various chronic diseases like, diabetes mellitus [1], Parkinson's disease [2], cardiovascular diseases [3], etc. Vitamin E helps to protect cell membrane against damage caused by free radicals and prevents the oxidation of low-density lipoprotein (LDL) cholesterol [4]. Vitamin E also plays a role in neurological functions and inhibition of platelet aggregation [5].

In spite of its widespread physiological importance and probable involvement in several chronic diseases, only few epidemiological studies are available for assessing role of vitamin E. A major hindrance in conducting such studies is the lack of availability of simple and cheap techniques for sample collection, preservation, and analysis.

Dried blood spot (DBS) refers to a blood sampling technique where little quantity of blood is spotted on a filter paper, dried, and preserved until analyzed [6]. This technique is well recognized in clinical laboratories for applications such as screening of inborn diseases like phenylketonuria in neonates. It has also been recently applied by many pharmaceutical companies in their drug development process, i.e., toxicokinetic and pharmacokinetic studies. The use of DBS in such studies is valuable

* Correspondence: archanachavan_83@yahoo.co.in
[1]Department of Pharmacology, Parul Institute of Pharmacy, Vadodara, Gujarat, India
Full list of author information is available at the end of the article

Table 1 System suitability parameters of developed bioanalytical method

Sr. no. (n = 6)	Retention time (min)		Resolution		Theoretical plate		Tailing factor	
	Vitamin E	ISTD	Vitamin E	ISTD	Vitamin E	ISTD	Vitamin E	ISTD
1.	10.225	13.580	-	5.519	4906.204	5741.899	1.033	1.262
2.	10.362	13.804	-	5.523	5117.783	5842.366	1.024	1.260
3.	10.324	13.579	-	5.494	4917.208	5743.893	1.032	1.150
4.	10.362	13.804	-	5.524	4928.743	5742.896	1.004	1.152
5.	10.225	13.581	-	5.521	4908.206	5516.296	1.019	1.155
6.	10.230	13.8	-	5.509	5111.704	5515.293	1.034	1.264
% RSD	0.6670	0.8895	-	-	-	-	-	-

as the sample volume required to be drawn every time is very less [7, 8].

Literature review suggests an LC-MS/MS method is reported for estimation of vitamin E from dried blood spot; however, the use of LC-MS/MS technique itself limits the utility of the method pertaining to the availability of such facility [9–11].

Therefore, in present study, we aimed to develop and validate a method for estimation of vitamin E from DBS using high-performance liquid chromatography (HPLC) method that may enhance the utility and acceptability of the method.

Methods

Instrument and analytical conditions

The HPLC analysis was carried out using Shimadzu (LC-20AD) isocratic pump with UV detector (SPD-20A) run on the LC-Solution software. The column used was Phenomenex C_{18} (250 × 4.6 mm, 5 μm), and detection was performed at 292 nm. Shimadzu (UV 1800) was used for UV visible analysis. Cooling microcentrifuge of REMI (RM 12 C) was used for centrifugation of samples.

Ethical approval

The need for ethical approval was waived as the study used left over blood samples from the laboratory.

Reagents and chemicals

Vitamin E and ascorbic acid were obtained from Sigma Aldrich (Mumbai, India). Triple distilled water was used for the HPLC experiment. HPLC grade methanol was purchased from SD Fine Chemicals Ltd. Other chemicals used were of AR grade and purchased from Sigma Aldrich (Mumbai, India). Whatman filter paper (grade 3, circles) 110 mm was obtained from GE Healthcare Life Sciences for blood spotting.

Preparation of standard stock solution

Accurately weighed 10 mg of vitamin E was transferred to 10-mL amber colored volumetric flask. Dissolved and diluted up to the mark with methanol (100% v/v) to give stock solution of 1000 μg/mL of vitamin E. From this solution, standard solutions with concentration of 60, 54, 45, 30, 9, 3, 1.25, and 0.625 μg/mL were prepared. The normal range of blood vitamin E level is 5.5 to 17 μg/mL. The concentration range of 0.625 to 60 μg/mL was selected to cover this range. The stock solution of 100 μg/mL alpha tocopheryl acetate was prepared and added to each test tube of vitamin E standard solution to yield final concentration of 30 μg/mL of alpha tocopheryl acetate as internal standard.

Preparation of spiked blood samples and DBS sample

A range of blood samples with concentration of 60, 54, 45, 30, 9, 3, 1.25, and 0.625 μg/mL were prepared by adding

Table 2 Specificity parameters for developed bioanalytical method

Sr. no.	Plasma sample	Interference at retention time of drugs		Interference at retention time of ISTD	
		Retention time (min)	Peak area	Retention time	Peak area
1.	A	NA	ND	NA	ND
2.	B	NA	ND	NA	ND
3.	C	NA	ND	NA	ND
4.	D	NA	ND	NA	ND
5.	E	NA	ND	NA	ND
6.	F	NA	ND	NA	ND

Table 3 Parameters for LLOQ samples of vitamin E

Extracted LLOQ samples				
Sample no.	Vitamin E		ISTD	
	Retention time (min)	Peak area	Retention time (min)	Peak area
1	10.233	42889	13.582	27852
2	10.258	42876	13.543	27853
3	10.231	42849	13.593	27869
4	10.232	42895	13.580	27859
5	10.255	42884	13.540	27843
6	10.258	42879	13.544	27849
Mean	10.244	42878.67	13.563	27854.16
SD (n = 6)	0.0137	16.0582	0.0238	8.9535
% CV	0.1342	0.0374	0.1756	0.0321

vitamin E stock solution of 1000 μg/mL. One hundred microliter of each blood sample was spotted on Whatman filter paper pretreated with 0.01% ascorbic acid. The same spot was spiked with 30 μL of 30 μg/mL internal standard solution. The cards were allowed to dry at room temperature and were stored at temperature below 4 °C.

Elution from DBS sample

On the day of analysis, the blood spot was cut and placed in a 10-mL centrifuge tube. One milliliter of 2.5% sodium dodecyl sulfate (SDS), and 2 mL ethanol was added to each tube containing DBS cutout. The tube was vortexed for 20 min and then kept at room temperature for 10 min. Hexane (3 mL) was added to each tube and vortexed for 10 min. After this, each tube was centrifuged at 5000 rpm for 10 min to separate layers. The hexane layers were transferred to individual beakers, followed by evaporation of solvent under vacuum. The residue was reconstituted with methanol (100% v/v) and analyzed using HPLC method.

Chromatographic conditions

Chromatographic analysis was performed on a Phenomenex C_{18} (250 × 4.6 mm, 5 μm) column. The mobile phase consisted of methanol to water (99:1% v/v), filtered through membrane filter, and degassed before pumping into HPLC system with following parameters. The flow rate was 1.4 mL/min. The detection wavelength was adjusted at 292 nm. Injection volume was 20 μL, and column was used at ambient temperature.

Method validation

The developed chromatographic method was validated for system suitability, specificity, sensitivity, accuracy, precision, recovery, and stability as per USFDA guidelines [12].

Results

Method development and optimization

After trials of several mobile phase compositions, methanol to water (99:1% v/v) at a flow rate of 1.4 mL/min was

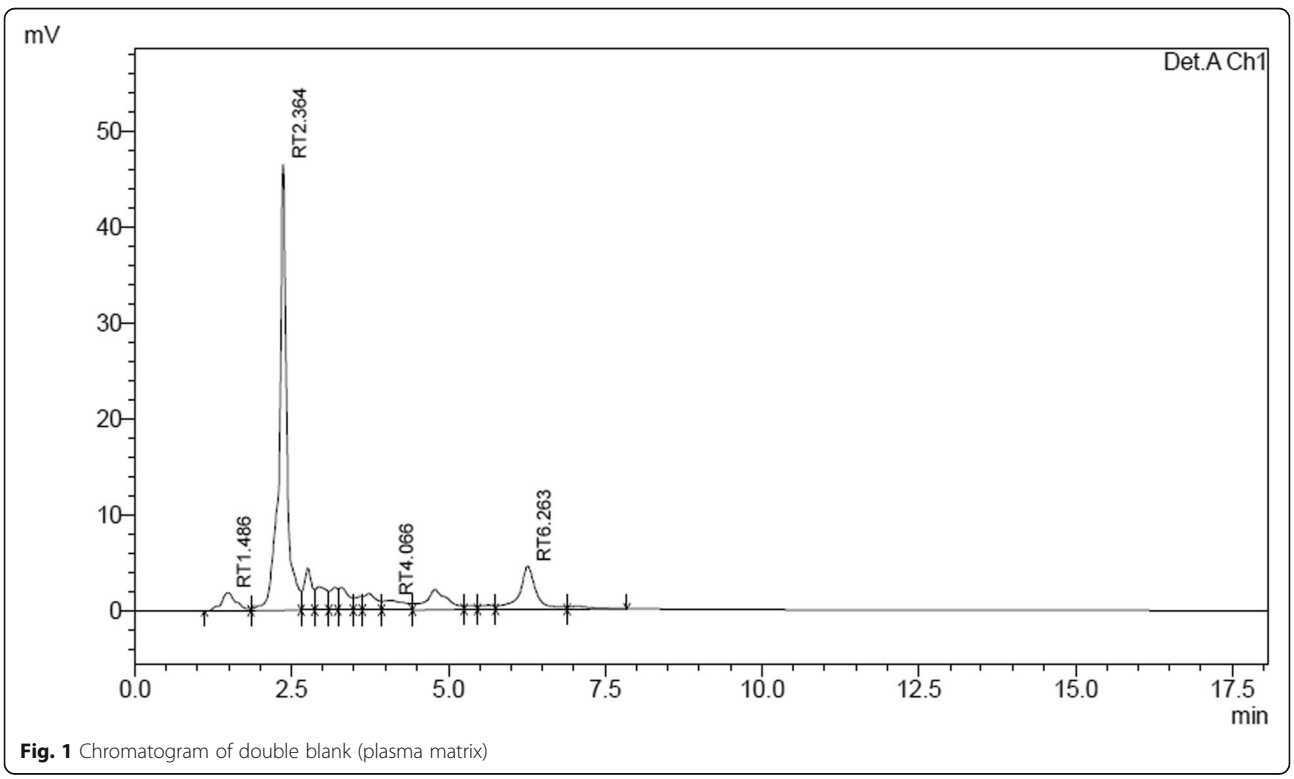

Fig. 1 Chromatogram of double blank (plasma matrix)

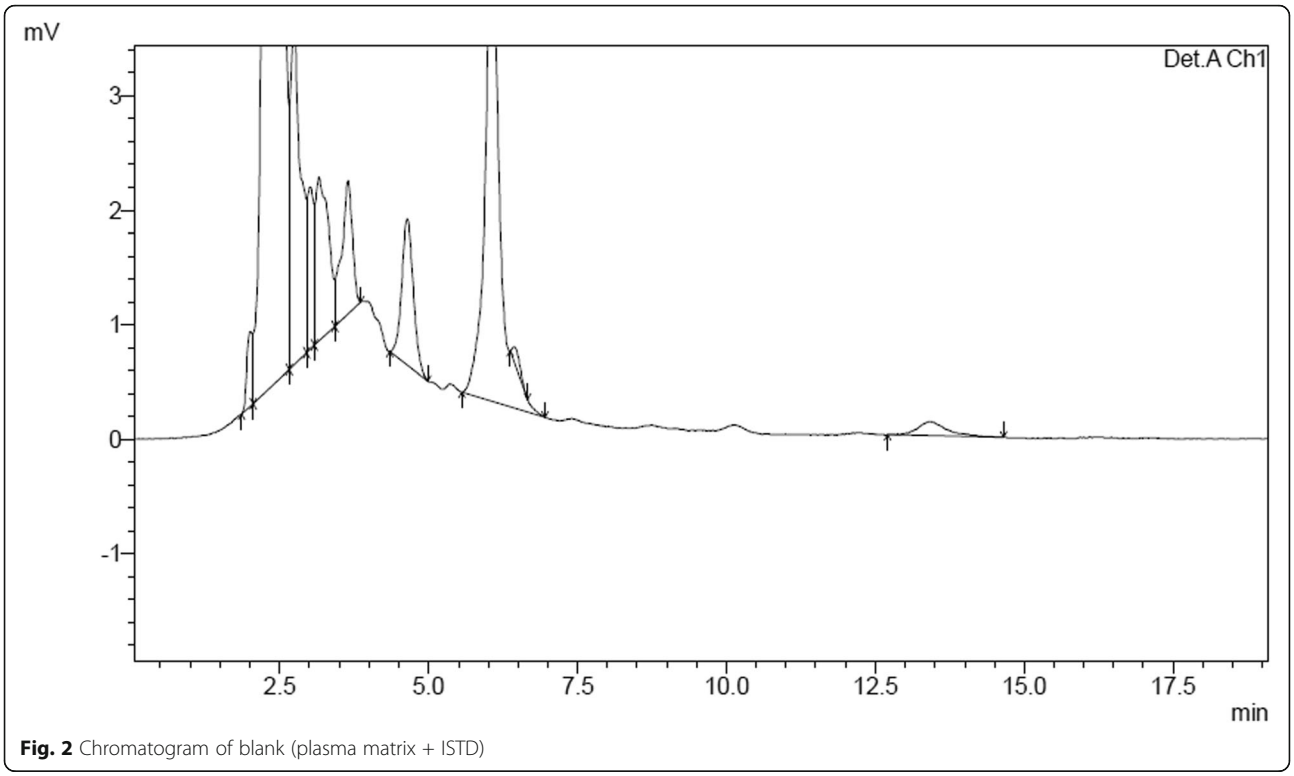

Fig. 2 Chromatogram of blank (plasma matrix + ISTD)

found to give best results with respect to peak separation, resolution, theoretical plate, and tailing factor. Detection wavelength of 292 nm was selected after scanning the standard solution of vitamin E over the range of 200–400 nm. Retention time for vitamin E and internal standard (alpha tocopheryl acetate) was 10.288 and 13.691 min respectively.

System suitability

For system suitability test, the mixture solution of 60 μg/mL vitamin E and 30 μg/mL internal standard was prepared. Parameters such as theoretical plate count, tailing factors, resolution, and reproducibility in retention time (RT) for six repetitions of vitamin E and internal standard were also explored for compliance with the system

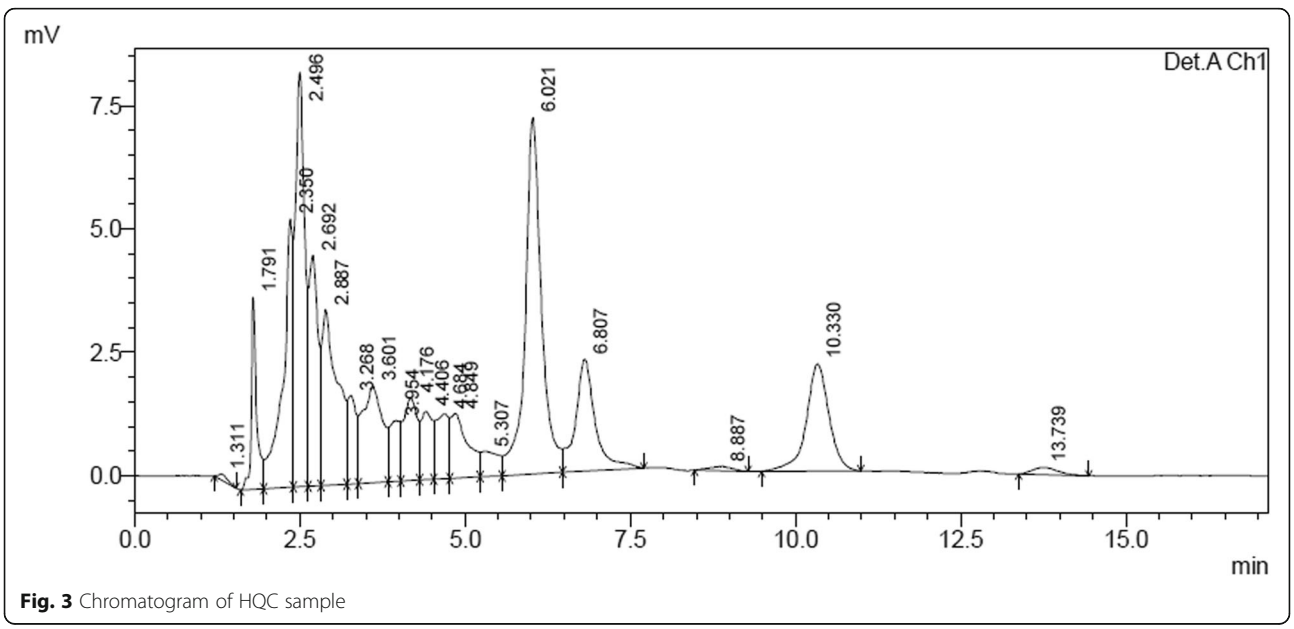

Fig. 3 Chromatogram of HQC sample

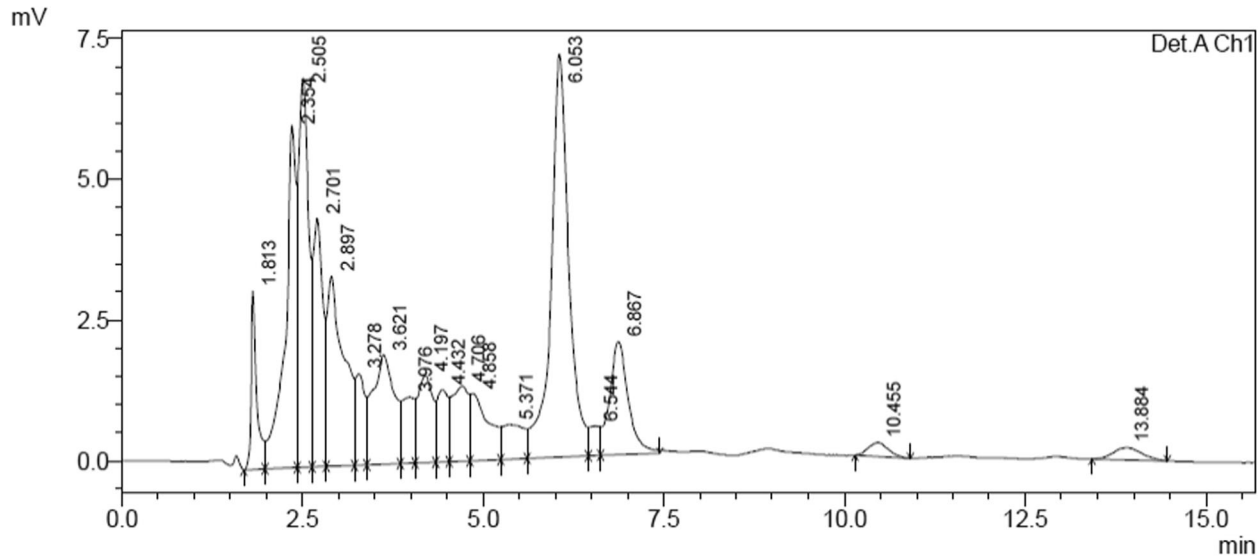

Fig. 4 Chromatogram of LLOQ sample

suitability test. The result of system suitability analysis is shown in Table 1.

Specificity

Specificity was checked for impact of matrix interference on the analysis of injected sample solution under optimized chromatographic conditions. The result of specificity is shown in the Tables 2 and 3.The chromatograms of double blank, blank, and non-zero sample (standard 1 and lower limit of quantification, LLOQ) are shown in Figs. 1, 2, 3, and 4.

Linearity and range

Various concentrations from standard and its working solution of vitamin E and internal standard (ISTD) were prepared, and calibration graph was plotted between the peak area ratio and concentration (μg/mL). Three times of upper limit of vitamin E normal range (20 μg/mL) in body was taken as STD 1 (high quality control, HQC), i.e., 60 μg/mL, while 0.625 μg/mL (20 μg/mL divided by 32) is taken as concentration of LLOQ. A range of eight concentrations were prepared including HQC and LLOQ. The result of linearity and range is shown in Table 4. Overlay chromatogram of vitamin E with internal standard is shown in Fig. 5, and linearity graph of area ratio vs concentration is shown in Fig. 6.

Accuracy

Accuracy of the method was established using recovery technique with added internal standard. Five replicates of lower quality control (LQC), medium quality control (MQC), and HQC each were prepared and analyzed. Intraday accuracy for vitamin E was observed from 101.591%, 103.147%, and 99.727% for MQC, HQC, and LQC respectively. Interday accuracy for vitamin E for MQC, HQC, and LQC was found to be 101.601 to 101.643%, 103.161 to 103.194%, and 99.895 to 100.454%. The result of percentage accuracy and percentage CV is shown in Table 5.

Table 4 Linearity parameters for developed bioanalytical method

Sr. no.	Concentration of vitamin E (μg/mL)	Area of vitamin E	Area of internal standard	$\frac{\text{Area of vitamin E}}{\text{Area of internal standard}}$ ($n = 3$)
1	60	321029	27883	11.5034 ± 0.01
2	54	285822	27874	10.2509 ± 0.0056
3	45	236809	27884	8.4593 ± 0.0577
4	30	176002	27882	6.3091 ± 0.0058
5	9	78832	27878	2.8210 ± 0.0115
6	3	48975	27870	1.7539 ± 0.0057
7	1.25	32550	27886	1.1738 ± 0.0057
8	0.625	25817	27860	0.9233 ± 0.0057

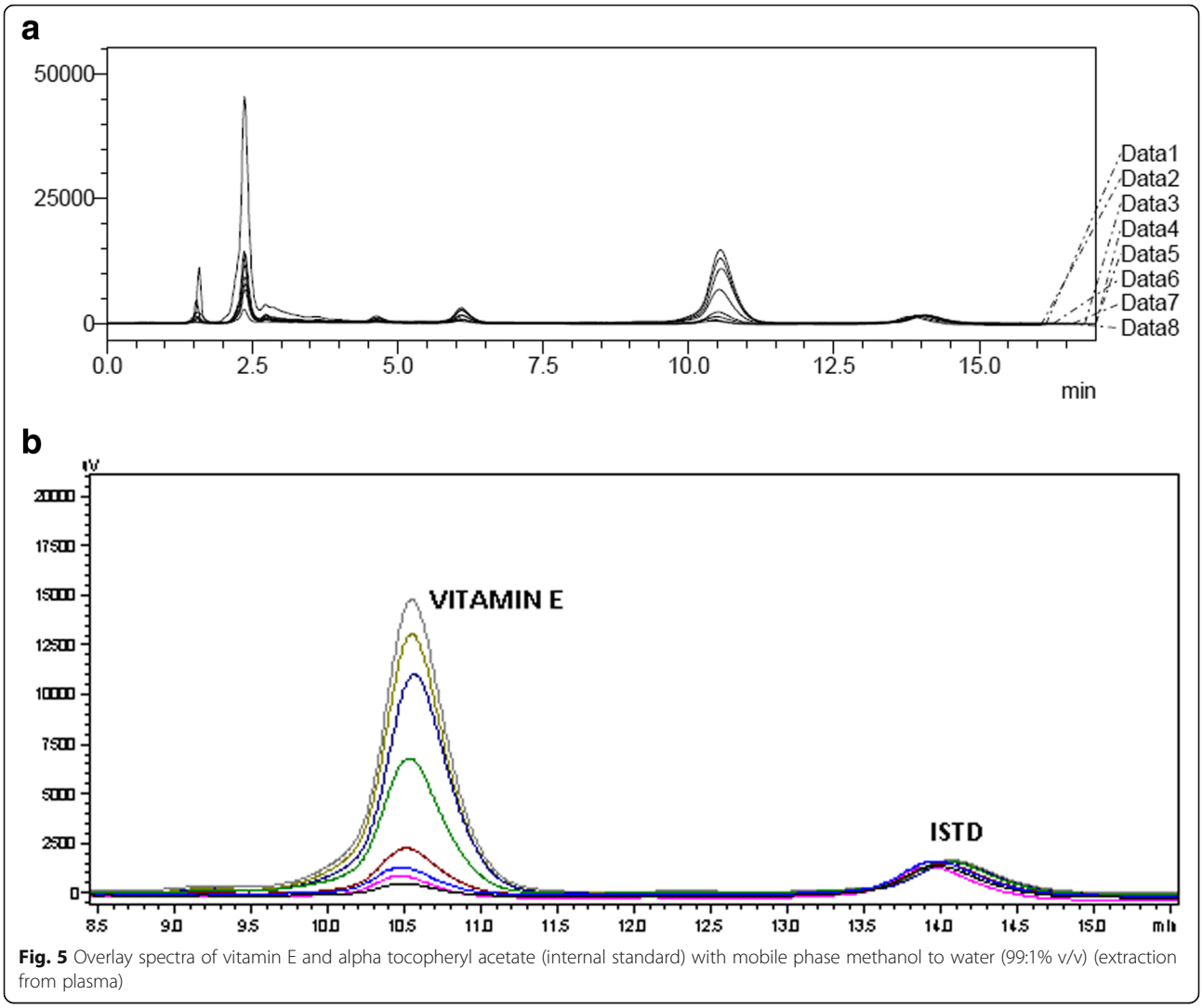

Fig. 5 Overlay spectra of vitamin E and alpha tocopheryl acetate (internal standard) with mobile phase methanol to water (99:1% v/v) (extraction from plasma)

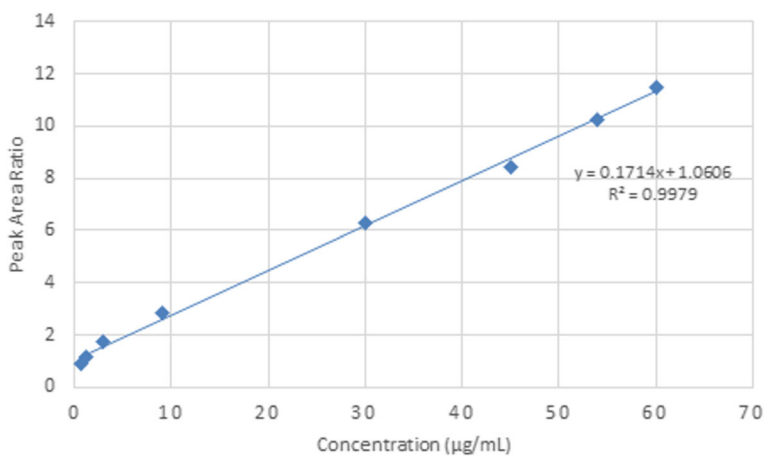

Fig. 6 Linearity curve of vitamin E

Table 5 Accuracy parameters for developed bioanalytical method

QC sample	Concentration (μg/mL)	Interday % accuracy	Intraday % accuracy			Mean ± SD (n = 3)	% CV
			Day 1	Day 2	Day 3		
LQC	1.875	99.727	99.895	100.454	100.174	100.174 ± 0.2795	0.2790
MQC	30	101.591	101.601	101.643	101.633	101.625 ± 0.0219	0.0215
HQC	45	103.147	103.161	103.194	103.182	103.179 ± 0.0167	0.0161

Precision

It is defined as the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of single homogenous volume of biological matrix. The precision of the developed method was studied by interday precision and intraday precision method. Five replicates of each concentration, i.e., LQC, MQC, and HQC were used for precision analysis. Intraday precision for high and medium QC samples was observed to be 0.0288 to 0.0191% which is within acceptance range of ± 15%, and for LQC sample, percentage of coefficient of variation (% CV) was found to be 0.0841% which is within acceptance range of ± 20%. Percentage CV for interday precision for HQC and MQC was found to be 0.01057–0.01923% and 0.01958–0.02484 % which is within acceptance range of ± 15%, and % CV for LQC sample was found to be 0.0501–0.1050% within the acceptance range of ± 20%. Percentage CV of intraday precision is shown in Table 6.

Recovery

It is defined as the detector response obtained from an amount of analyte added and to that extracted from the biological matrix. Repeatability was checked by injecting each concentration of six determinations such as LQC, MQC, and HQC respectively. Percentage recovery of vitamin E for LQC, MQC, and HQC was found to be 95.7713%, 91.1820%, and 93.0659%. The result of recovery is shown in Table 7.

Stability

Different stability studies for vitamin E, i.e., short-term stability, freeze thaw stability, long-term stability, and stock solution stability were performed as per USFDA guideline, and observed data are given in Table 8 which was observed within 15% for LQC and HQC acceptance criteria.

Developed method was validated as per USFDA guideline, and all parameters such as accuracy, precision, selectivity, recovery, and stability study were as per acceptance criteria.

Discussion

In our study, we used Whatman filter paper (grade 3, circles, 110-mm diameter) as a matrix for blood spotting. Several studies are available which have shown suitability of filter papers of specific grade for collection of blood samples in DBS technique [13, 14]. The use of filter papers instead of specialized discs makes the method more approachable for many researchers, where supply of specialized discs may be limited.

As per USFDA guideline for validation of bioanalytical method, the method is specific as no interference was found for vitamin E or ISTD with the biological matrix and percentage CV for vitamin E (LLOQ), and internal standard was found to be within the acceptance range. The method was found to be linear over the concentration range of 0.625 to 60 μg/mLwith R^2 value of 0.9979. Intraday accuracy and interday accuracy for vitamin E is within the acceptance range of % CV ± 15% and ± 20% for MQC, HQC, and LQC respectively; hence, developed method is accurate. Method is reproducible, as intraday precision and interday precision for high and medium QC samples is within the acceptance range of ± 15%, and for LQC sample, % CV was found to be within acceptance range of ± 20%. Good recovery was found within the range of 91.1820 to 95.7713% with % CV 0.2074 to 0.3971 for all QC samples. The stability study data for vitamin E, i.e., short-term stability, freeze thaw stability, long-term stability, and stock solution stability were observed within acceptance criteria of 15% for LQC and HQC.

The existing method for analysis of vitamin E by DBS involves use of LC-MS technique. Sensitivity of LC-MS

Table 6 Intraday and Interday precision parameters for developed bioanalytical method

QC samples	Concentration (μg/mL)	Precision					
		Intraday			Interday		
		Mean	SD (n = 5)	% CV	Mean	SD (n = 5)	% CV
LQC	1.875	2.284	0.0019	0.0841	2.2833	0.0005	0.0252
MQC	30	7.743	0.0014	0.0191	7.7416	0.0005	0.0074
HQC	45	10.781	0.0031	0.0288	10.782	0.0010	0.0092

Table 7 Percentage recovery of vitamin E for developed bioanalytical method

QC samples	Vitamin E		
	Concentration (μg/mL)	Mean recovery ± SD (n = 6)	% CV
LQC	1.875	95.7713 ± 0.2572	0.2685
MQC	30	91.1820 ± 0.3620	0.3971
HQC	45	93.0659 ± 0.1930	0.2074

method is higher as compared to that of our newly developed method. However, the cost of analysis and availability of LC-MS instrument are a major limitation of the technique. The developed method is cost effective, and HPLC instrument is widely available.

Conclusion

Method was developed in mobile phase methanol to water (99:1% v/v), flow rate 1.4 mL/min at 292 nm with alpha tocopheryl acetate as an internal standard. Developed method was validated as per USFDA guideline. The developed method was found to be highly accurate, specific, and precise. Stability studies like short term, long term, freeze thaw, and stock solution were also performed, and analyte was found to be stable. Thus, it can be concluded that vitamin E can be estimated from dried blood spot by RP-HPLC method.

Implication for health practice and research

Vitamin E being important lipid phase antioxidant mechanism, its derangement may be one of the factors in many recent disease breakthroughs. However, due to unavailability of suitable method, population-based epidemiological studies are missing. The method developed by us can be a valuable tool in field-based epidemiological studies, where immediate processing of the sample is not possible. The blood spot collection is a subject friendly, compact, and easy to store method as compared to collection of wet samples. Moreover, RP-HPLC analysis makes it economic and widely adaptable.

Table 8 Stability parameters for developed bioanalytical method

Stability study	QC samples	Vitamin E	
		Mean area ratio of fresh sample ± SD (n = 5)	Mean area ratio of stability sample ± SD (n = 5)
Short-term stability	**LQC**	2.2726 ± 0.0020	2.254 ± 0.0015
	HQC	10.7822 ± 0.0023	10.7644 ± 0.0022
Freeze thaw stability	**LQC**	2.274 ± 0.0015	2.2068 ± 0.0076
	HQC	10.7812 ± 0.0043	10.7288 ± 0.0039
Long-term stability	**LQC**	2.2804 ± 0.0099	2.1984 ± 0.0110
	HQC	10.7806 ± 0.0060	10.6876 ± 0.0090
Stock solution stability (at RT)	**LQC**	2.284 ± 0.0069	2.277 ± 0.0079
	HQC	10.786 ± 0.0069	10.777 ± 0.0058
Stock solution stability (at 2–8 °C)	**LQC**	2.2836 ± 0.0089	2.2704 ± 0.0081
	HQC	10.7866 ± 0.0082	10.7702 ± 0.0023

Abbreviations

% CV: Percentage of coefficient of variation; HPLC: High-performance liquid chromatography; HQC: High quality control; ISTD: Internal standard; LDL: Low-density lipoprotein; LLOQ: Lower limit of quantification; LQC: Lower quality control; MQC: Medium quality control

Acknowledgements

None

Authors' contributions

AN conceptualized the project and gave technical inputs in conducting the study and preparing manuscript. VP performed the study and prepared manuscript. FT gave technical inputs in conducting the study. All authors have read and approved the manuscript.

Funding

This project was funded by Parul University. The instrument, chemicals, and operational costs were borne by Parul University.

Availability of data and materials

All data and material is available upon request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Department of Pharmacology, Parul Institute of Pharmacy, Vadodara, Gujarat, India. [2]Department of Quality Assurance, Parul Institute of Pharmacy, Vadodara, Gujarat, India.

Received: 31 August 2019 Accepted: 3 March 2020
Published online: 19 March 2020

References

1. Milman U, Blum S, Shapira C (2008) Vitamin E supplementation reduces cardiovascular events in a subgroup of middle-aged individuals with both type 2 diabetes mellitus and the haptoglobin 2-2 genotype: a prospective double-blinded clinical trial. Arterioscler Thromb VascBiol 28:341–347
2. Morens DM, Grandinetti A, Waslien CI, Park CB, Ross GW, White LR (1996) Case-control study of idiopathic Parkinson's disease and dietary vitamin E intake. Neurology 46:1270–1274
3. Sesso HD, Buring JE, Christen WG (2008)Vitamins E and C in the prevention of cardiovascular disease in men: the Physicians' Health Study II randomized controlled trial. JAMA300:2123-2133.
4. Dror DK, Allen LH (2011) Vitamin E Deficiency in Developing Countries. Food NutrBul 32:124–143
5. Stocker R (2007) Vitamin E. Novartis Found Symp 282: 77-87.
6. Suva MA (2014) A brief review on dried blood spots application drug development. J Pharm Bio Sci 1:17–23
7. Spooner N, Lad R, Barfield M (2009) Dried blood spots as a sample collection technique for the determination of pharmacokinetics in clinical studies: considerations for the validation of a quantitative bioanalytical method. Anal Chem 81:1557–1563
8. Freeman JD, Rosman LM, Ratcliff JD, Strickland PT, Graham DR, Silbergeld EK (2018) State of the science in dried blood spots. ClinChem 64:656–679
9. Zaspel BJ, Csallany AS (1983) Determination of alpha-tocopherol in tissues and plasma by High-performance liquid chromatography. Anal Biochem 130:146–150

10. Bieri JG, Teresa J, Toiiver BS, George L (1979) Simultaneous determination of alpha-tocopherol and retinol in plasma or red cells by high pressure liquid Chromatography. Am J ClinNutr 32:2143–2149
11. Midttun Ø, Ueland PM (2011) Determination of vitamins A, D and E in a small volume of human plasma by a high-throughput method based on liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 25:1942–1948
12. Bioanalytical method validation, guidance for industry (2001) US Department of Health and Human Services, Rockville.
13. World Health Organization. (2016). Malaria microscopy standard operating procedures. https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf Accessed 21 Sept 2018.
14. Kadjo AF, Stamos BN, Shelor CP, Berg JM, Blount BC, Dasgupta PK (2016) Evaluation of amount of blood in dry blood spots: ring-disk electrode conductometry. Anal Chem 88:6531–6537

Publisher's Note