Possible Interaction of Water-Soluble Vitamins with Nitric Oxide and Its Significance – Evidence from Reversed Phase-High Pressure Liquid Chromatography (RP-HPLC)

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Abstract **- Water soluble vitamins (B series and C) are physiologically significant biomolecules since one of the major roles for some of them is to scavenge metabolically harmful reactive oxygen species (ROS) produced in living beings. Nitric oxide (NO), a naturally produced gaseous and reactive biomolecule, also serves as an antioxidant in cell metabolism depending majorly on its endogenous concentration. In view of these apparently similar biochemical roles of water-soluble vitamins and NO, authors have attempted to investigate possible interaction between them. In this regard, evidence has been generated from RP-HPLC coupled with UV detector. Interestingly, all water-soluble vitamins, except vitamin B2 (riboflavin) and B5 (calcium D-pantothenate), exhibited noteworthy changes in their respective retention times and/ or significant changes in the intensity of peak absorbance upon interaction with equimolar concentration of NO donor (Diethylenetriamine NONOate; DETA). Vitamin B7 (D-Biotin) and B6 (pyridoxine hydrochloride) showed a very significant increase in their respective peak absorbance (112.5 and 11.33-fold, respectively) at 247 nm. These observations provide new evidence for possible interaction of NO with some of the water-soluble vitamins and offer novel mechanisms of action of water-soluble vitamins as ROS scavengers in biological systems.**

Index Terms **- Water-soluble vitamins, Nitric Oxide, RP-HPLC, ROS scavenging .**

I.INTRODUCTION

Vitamins are a group of organic compounds required in limited amounts by living organisms for multiple biochemical reactions. In natural products, vitamins are present either in free state or are chemically bonded with other biomolecules [1,2]. Water soluble vitamins constitute a group of chemically heterogenous organic compounds which include neutrals, acids, bases and zwitterions. They include vitamin B_1 (thiamine hydrochloride), B_2 (riboflavin), B_3 (niacin), B_5 (Calcium-D-pantothenate), B_6 (pyridoxine hydrochloride), B_7 (D-Biotin), B_9 (folic acid), B_{12} (cyanocobalamin) and C (L-Ascorbic Acid). Fat soluble vitamins includes vitamin A (retinol), D (calciferol), E (tocopherol) and K (phylloquinone). Vitamins are not synthesized by the human body and are taken up from food. Two exceptions are vitamin B_3 (nicotinic acid) which, in active form (NADH/NADPH), can be synthesized from tryptophan, and vitamin D_3 (cholecalciferol), synthesized from dehydrocholesterol. Water soluble vitamins usually function as precursors of coenzymes and prosthetic groups of enzymes.

Thiamine (vitamin B_1) occurs in natural products in its free form as the mono-, di- and triphosphoric esters, as thiamine disulphide, and sometimes in other sulphur-containing compounds. In pharmacological preparations, thiamine is used as a hydrochloride. Thiamine hydrochloride (B_1) is derived from a substitute pyrimidine and a thiazole, which are coupled by a methylene bridge [3]. It is rapidly converted to its active form, Thiamine Pyrophosphate (TPP) in brain and liver, by the activity of the enzyme - thiamine diphosphotransferase. Riboflavin (B_2) contains an isoalloxazine ring attached to ribitol. It is

the precursor of coenzymes - flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). The enzymes that require FMN or FAD or are called flavoproteins. Several flavoproteins contain metal ions and are called metalloflavoproteins. These enzymes are involved in a wide range of redox reactions. During these enzymatic reactions, the reduced forms of FMN and FAD, i.e., $FMNH_2$ and $FADH_2$, respectively, are formed. Both nicotinic acid and nicotinamide occur in nature (vitamin B_3). Free acid has the same biological activity as the amide. Nicotinamide is a component of some enzymes. Niacin (nicotinic acid, vitamin B_3) is a pyridine derivative. The amide form of niacin is known as nicotinamide. It can be synthesized endogenously from tryptophan. Both nicotinic acid and nicotinamide are required for the biosynthesis of the active forms of vitamin B_3, namely nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$). Both NAD^+ and $NADP^+$ function as cofactors for a number of dehydrogenases. They are present in the living system both in oxidised and reduced state. Both undergo reduction of the pyridine ring by accepting hydride ion (H^-). Calcium-D-pantothenate (vitamin B_5) occurs in plants and animals both in free state and in bonded form, such as co-enzyme A. Pantothenic acid is derived from beta-alanine and is required for the synthesis of coenzyme–A (CoA). Vitamin B_6 constitutes pyridoxal, pyridoxamine and pyridoxine hydrochloride, all of which are pyridine derivatives and are efficiently converted into the biologically active form of vitamin B_6, i.e., pyridoxal phosphate. Pyridoxal phosphate serves as a cofactor in transamination reactions. Folic acid (vitamin B_9) occurs in nature both in free form and as a derivative, in which glutamic acid radical is linked (as in peptides) to the pteroyl structure of folic acid. D-Biotin (vitamin B7) is also present in traces in living systems, both in free form and as co-enzymes. D-Biotin (B7) is involved in carboxylation reactions. Folic acid (B_9) is reduced within the cells to tetrahydrofolate (THF), which functions to carry out transfers of one carbon units such as methyl ($-CH_3$), methylene ($-CH_2$), formyl (-CHO) or formimino ($CH_2=N-$) groups. Cyanocobalamin (vitamin B_{12}) occurs in nature in much less quantity relative to other B vitamins. It is composed of complex tetrapyrrole ring structure (corrin ring) with a cobalt ion in the centre. L-Ascorbic acid (vitamin C) is derived from glucose via uronic acid pathway. It serves as a reducing agent in a number of biochemical reactions and is also a cofactor in the hydroxylation of proline. L-Ascorbic acid (vitamin C) freely occurs in nature and it also combines with proteins.

Recent reports have suggested that some vegetables and fruits rich in nitrate can provide a physiological substrate for reduction to form nitric oxide (NO). These include green leafy vegetables such as lettuce, spinach and beetroot. Incidentally, these vegetables are also rich in one or more of the vitamins. In the acidic environment of the stomach, part of the swallowed nitrate is protonated to nitrous acid which then decomposes to a variety of nitrogen oxides, including NO [4,5]. There has been immense interest in linking the chemistry of NO to its biological effects. NO has high diffusion constant and can travel significant distance in a very short time, down a concentration gradient. NO forms additional bioactive adducts, such as *S*-Nitrosothiols and *N*-Nitrosoamines, upon reaction with protein thiols and amines, respectively in dietary products. NO production in stomach is also greatly enhanced in presence of L- acid (vitamin C). Some of the NO that escapes protonation in the acidic milieu of the stomach, reaches blood stream and muscles to exert its effect directly. High affinity of NO for transition metal centres, particularly iron, and its rapid reaction with oxygen - derived free radicals can explain many of the biological properties of NO. Since iron in many haemoproteins is redox active, it can participate in one-electron oxidation-reductions of NO.

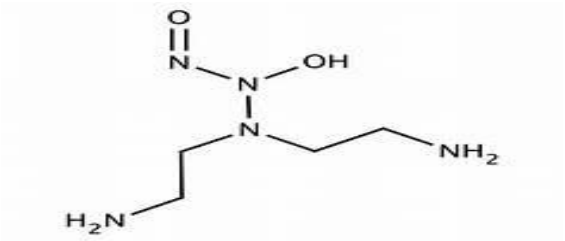
Due to its high reactivity, NO interacts with a variety of biomolecules. At low concentrations, NO reacts with organic molecules and oxygen species while high NO concentrations would lead to nitrogen oxides reactions. Thus, the pro-oxidant or antioxidant behaviour of NO is determined by the available concentration of NO [6,7]. Reaction of NO with oxygen yields nitrogen dioxide, a potent oxidant. NO is a small, uncharged molecule that contains an unpaired electron as it has odd number of electrons. NO is a free radical and can stabilize its unpaired electron by two mechanisms:1. Reaction with species containing other unpaired electrons (thus pairing up the two lone electrons) and, 2. Interaction with *d*-orbitals of transition metals, particularly iron. Reactions of NO with heme in iron-containing enzymes/proteins are, by now, quite well investigated

[8]. It is generally believed that all free radicals are unstable and highly reactive. NO is, however, chemically stable and much less reactive with non-radical species than the hydroxyl radical but more reactive than lipid peroxyl radicals [9]. The property of possessing an unpaired electron is referred as paramagnetism, and it is this property that determines the chemical reactivity of NO with other radical species. The initial reaction of NO with non-metals under biological conditions is the oxidation of cellular nucleophiles and formation of relatively stable nitrite and nitrate anions [10]. Although NO is not an oxidant, nitrite definitely is and it reacts rapidly with many molecules 'rich' in electrons (nucleophiles) to extract one electron. NO also reacts with one electron reduced form of oxygen i.e., superoxide, resulting in the formation of peroxynitrite ($ONOO^-$) which is a moderately strong two-electron oxidant.

In view of the above-stated reactive and antioxidative features of most of the water-soluble vitamins and NO, present investigation has been undertaken to examine possible interaction of NO with water soluble vitamins. The work has been carried out by co-incubation of an established NO donor (Diethylenetriamine NONOate; DETA) with equimolar concentrations of individual water-soluble vitamins. Pure vitamins and those co-incubated with NO donor were subjected to RP-HPLC. Differences in retention profiles and UV absorbance properties of vitamins (pure and with NO) were analysed to assess possible interactions of water-soluble vitamins with NO. The work provides new reports on NO-vitamins interactions and discusses their potential implications to understand the antioxidative roles of vitamins and NO.

II. MATERIALS AND METHODS

DETA NONOate as NO donor: Diethylenetriamine NONOate {(Z)- 1- [N- (2-aminoethyl) -N - (2 - ammonioethyl) amino] diazen-1-ium-1,2-diolate; MF: $C_4H_{13}N_5O_2$; MW: 163.2} is an established NO donor which is highly soluble in water and releases free amine and NO upon dissociation in a pH-dependent manner following first order kinetics [11,12]. It can be stored under nitrogen for up to one year. DETA liberates two moles of NO per mole of parent compound. The half-life of NONOate in 0.1 M phosphate buffer (pH 7.4) solution is 20 hrs and 56 hrs at 37˚ C and 25˚ C, respectively. The crystals are sensitive to moisture. For the present work DETA NONOate was procured from Cayman Chemical, MI, USA (Product no. 82120).



Preparation of stocks for NO and Vitamins: Stock solutions of water-soluble vitamins of the B series (B_1, B_2, B_3, B_5, B_6, B_7, B_9 and B_{12}) and vitamin C were prepared in HPLC grade water at a uniform concentration (2mg/ml). From the prepared stock solutions, dilutions of 100 µg/ml were prepared (Table I). After calculating the molar concentration for each vitamin individually in 100 µg/ml, equimolar concentration of DETA was added to the respective vitamin solutions for further analysis.

TABLE I. Effective concentrations of individual vitamins for RP-HPLC

S. No.	Vitamins	Effective concentrations (µM) in 100 µg/ml
1.	B1 (Thiamine Hydrochloride)	296.5
2.	B2 (Riboflavin)	265
3.	B3 (Nicotinic Acid)	813
4.	B5(Calcium D-pantothenate)	210
5.	B6 (Pyridoxine Hydrochloride)	485
6.	B7 (D- Biotin)	410
7.	B9 (Folic Acid)	227
8.	B12 (Cyanocobalamin)	73.8
9.	C (L-Ascorbic Acid)	568

From the prepared stock solutions of individual vitamins alone or in combination with DETA, 100 µg/ml of each was injected for RP-HPLC. Table I shows effective concentrations of individual vitamins used for RP-HPLC analysis. The focus of this work required loading of each water-soluble vitamin for RP-HPLC analysis one at a time and its possible interaction with NO in solution.

HPLC of Vitamins: Reverse phase HPLC was performed on HPLC system from Waters, USA. Running conditions employed Sunfire C18 column (dimensions: 4.6 X 250 mm, 5 µm), ambient room temperature (25 C), flow rate of 0.8 ml/min, mobile phase A consisting of 5.84 mM Hexane- 1 sulphonic

acid: Acetonitrile (95:5) containing 0.1% Triethylamine (pH 2.5, obtained using ortho-phosphoric acid), mobile phase B consisting of 5.84 mM Hexane- 1 sulphonic acid: Acetonitrile (50:50) containing 0.1% Triethylamine (pH 2.5) D. Gradient conditions: Binary elution gradient was followed. To begin with, at 0 min, concentration of mobile phase was 100%. After 20 min, mobile phase A was reduced from 100 to 50 %. Gradient run time was 22 min. Injection volume was 50 µl full loop injection. Detector was photodiode detector (PDA), used at wavelengths 210, 247, 270 and 290 nm. Data was acquired and processed using WatersEmpower ™ 2 software. All major and minor changes in the retention time (RT) profiles of pure vitamins alone or in combination with NO were assessed by UV detector at one or more wavelengths close to or at the established peak absorbance wavelengths.

All analyses were performed at least thrice.

III. RESULTS

Since the UV detector responses of various vitamins are expected to show differences, the signal of each vitamin was monitored in present work at different wavelengths specific to analytes as shown in Table II.

Table II Detection of water-soluble vitamins by UV detector during RP-HPLC analysis

Water soluble vitamins	Wavelengths of detection used earlier (nm)*	Wavelengths of detection used in the present work (nm)
B1 (Thiamine hydrochloride)	248, 254, 270	247, 290
B2 (Riboflavin)	254, 268, 270	247, 270
B3 (Nicotinic Acid)	254, 260, 270	260
B5 (Calcium-D-Pantothenate)	197, 210, 220	210, 230, 247
B6 (Pyridoxine hydrochloride)	210, 280, 290	247, 290
B7 (D-Biotin)	205	210, 247
B9 (Folic Acid)	254, 258, 280, 290, 345, 350	247
B12 (Cyanocobalamine)	254, 360	360
C (L-Ascorbic Acid)	225, 245, 254, 260, 265, 270	247, 270

*According to ThermoFisher Scientific Technical Note 7248

This procedure was adopted to get maximum sensitivity of detection of various vitamins which vary significantly in their chemical nature. Vitamins were separated to the baseline and eluted as sharp peaks within 22 min. The separation times were reproducible. DETA (NO donor) in aqueous solution at a concentration of 500 µM, when passed through RP-HPLC column and allowed to migrate exactly under the conditions used for the migration of individual water-soluble vitamins, did not exhibit any absorbance peak at any of the four UV detection wavelengths chosen (210, 247, 270 and 290 nm) during the 22 min run time, except a very minor jump from basal level at 210 nm between 2.5 to 5 min (Fig. 1). These observations clearly strengthen the view that if any changes are observed in the peak profiles (change in retention time, additional peak or peak area) of the different vitamins upon interaction with NO donor, they will probably reflect a possible chemical interaction of NO with the said vitamin.

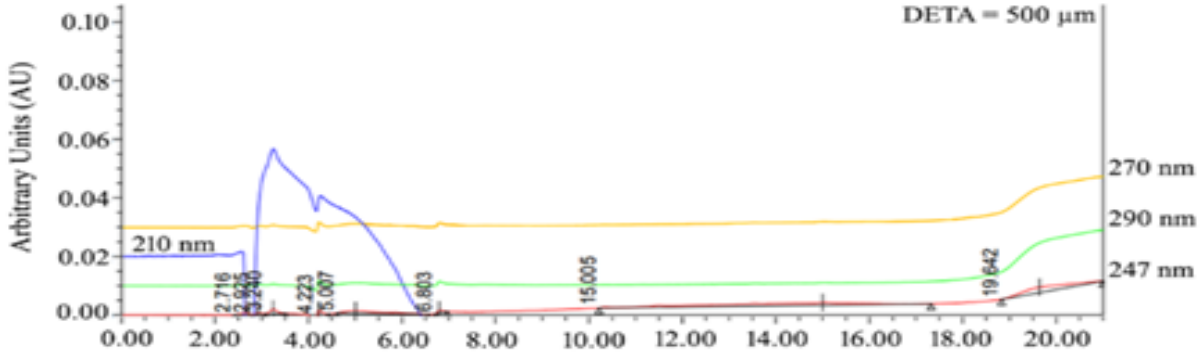


Fig. 1 HPLC analysis of NO donor, DETA (500 µM) at 200, 247, 270 and 290 nm.

The RT profiles and UV detection responses of various water-soluble vitamins exhibited following novel alterations upon interaction with NO in solution: In case of Riboflavin (Vitamin B_2) minor UV absorbance is evident at 247 nm while major peak is detected at 270 nm. There is no change in λ max (270 nm) and the extent of absorbance at λ max of 270 nm in presence of NO donor (DETA) (Fig. 2).

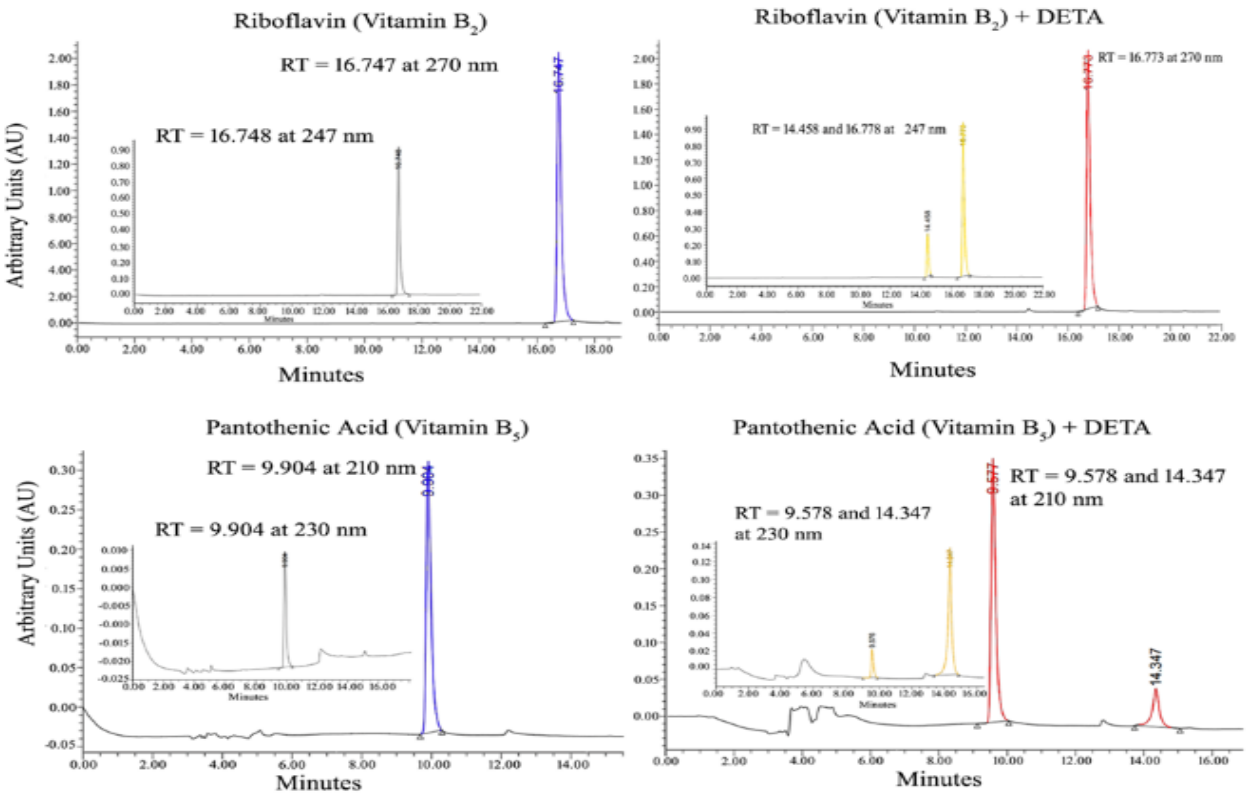


Fig. 2 HPLC analysis of Riboflavin (Vitamin B_2) and Calcium-D-Pantothenate (Vitamin B_5), in the absence as well as presence of NO donor, DETA, exhibit shifts in their RT peaks under the influence of nitric oxide (NO) provided as DETA (NO donor).

Minor absorbance observed at 247 nm, however, exhibits an additional (minor) peak at 14.458 (% peak area = 18.77 %). Thus, nitric oxide does not seem to chemically interact with riboflavin**.** Interaction of NO with calcium-D-pantothenate (vitamin B_5) is not evident since the major peak at 210 nm shows a minor change in RT from 9.904 to 9.577 with a slight increase in absorbance. All the other peaks at 210 and 230 show a similar trend with marginal change upon incubation of vitamin B5 with DETA (Table III).

Table III Impact of co-incubation of NO donor (DETA) with various water-soluble vitamins, Riboflavin (B_2) and Calcium-D-Pantothenate (B_5) (individually), as evident from HPLC analysis

S.No.	Vitamin		Wavelength (nm)	Retention time (min)	Peak Area (%)	Absorbance (AU)	Fold-change due to NO
1.	Riboflavin (B_2)	Peak 1					
		Control	247	16.748	100	0.92	-
		(+) NO		14.458	18.77	0.26	+3.5
				16.773	81.23	0.94	-
		Peak 2					
		Control	270	16.747	100	2.05	-
		(+) NO		16.773	100	2.05	-
2.	Calcium-D-Pantothenate (B_5)	Peak 1					
		Control		9.904	100	0.32	-
		(+) NO	210	9.577	81.76	0.35	-
				14.347	18.24	0.04	-
		Peak 2					
		Control		9.904	100	0.08	-
		(+) NO	230	9.576	12.08	0.02	- 4
				14.347	87.92	0.13	+1.6
		(+) NO	247	14.462	29.84	0.25	-
				15.411	70.16	0.54 ↑	+ 2.35

Interaction of nitric oxide with pantothenic acid is, thus, not evident. Thiamine hydrochloride (Vitamin B_1) majorly showed absorbance at 290 nm (% peak area = 100%) with a minor peak absorbance at 247 nm. Upon co-incubation with equimolar concentration of DETA (NO donor), however, the major peak detected at 290 nm almost disappeared at an RT close to that of control i.e., at 15.774 min (Fig. 3). Instead, the reaction product showed noteworthy absorption at 247 nm with an RT of 19.622 (% peak area = 84.46%) with a very minor absorbance peak at 15.770 (% Area = 15.54**).** A possible interaction of thiamine hydrochloride with nitric oxide (NO) is thus evident by way of a major shift in RT of the reaction product is thus evident at 247 nm. In case of Nicotinic acid (Vitamin B_3) **t**here is a noteworthy shift in the RT for both peak 1 (minor) and peak 2 upon its co-incubation with NO (equimolar) (Table IV).

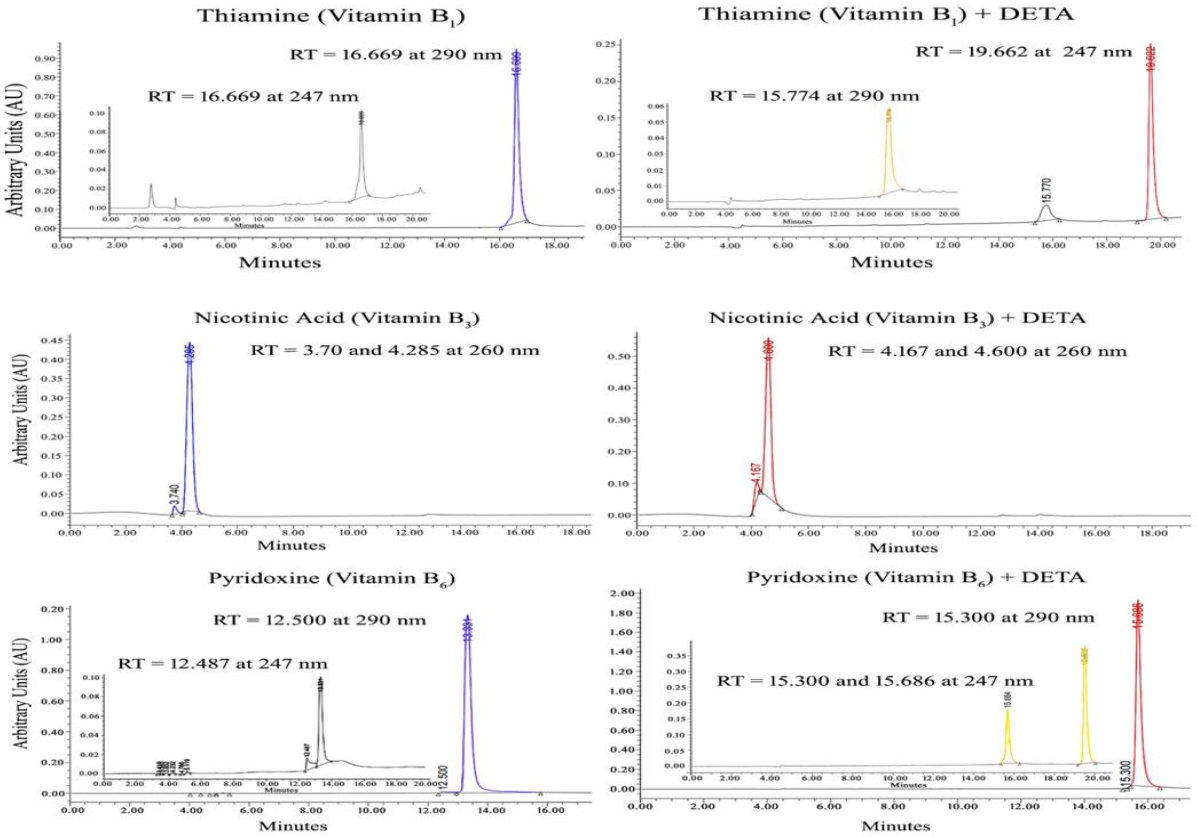


Fig. 3 HPLC analysis of Thiamine hydrochloride (Vitamin B_1), Nicotinic acid (Vitamin B_3) and Pyridoxine hydrochloride (Vitamin B_6) in the absence as well as presence of NO donor, (DETA), exhibit significant shifts in their respective Retention times (RT in minutes) after incubation with Nitric oxide donor (DETA)

Table IV Impact of co-incubation of NO donor (DETA) with various water-soluble vitamins, B_1, B_3 and B_7 (individually), as evident from HPLC analysis.

S.No.	Vitamin		Wavelength (nm)	Retention time (min)	Peak Area (%)	Absorbance (AU)	Fold-change due to NO
1.	Thiamine hydrochloride (B_1)	Peak 1					
		Control	247	16.609	100	0.12	-
		(+) NO		19.622	84.46	0.25	+2
				15.770	15.54	0.02	-
		Peak 2					
		Control	290	16.609	100	0.95	
		(+) NO		15.774	100	0.06	-16
2.	Nicotinic acid (B_3)	Peak 1					
		Control	260	3.740	2.76	0.02	-
		(+) NO		4.167	6.81	0.12	+6
		Peak 2					
		Control	260	4.285	97.24	0.45	-

		(+) NO		4.600	93.19	0.58	+1.28
3.	Pyridoxine hydrochloride (B_6)	Peak 1					
		Control	247	12.487	14.84	0.015	-
				13.331	82.61	0.10	-
		(+) NO	247	15.684	32.70	0.17 ↑	+11.33
				19.506	67.30	0.38 ↑	+2.23
		Peak 2					
		Control	290	13.331	98.73	1.15	-
		(+) NO		15.686	99.94	1.90 ↑	-

So also, the extent of absorbance at 260 nm is marginally altered. NO interaction with NA is, thus, not evident. Pyridoxine hydrochloride (Vitamin B_6) exhibits a dramatic shift in RT and absorbance at 290 nm upon interaction with NO (Table IV). Similar shift in RT and absorbance towards upper side is also evident for the two minor peaks detected at 247 nm. Nitric oxide interaction with pyridoxine hydrochloride is, thus, evident. A very strong impact of NO donor (DETA) upon incubation with vitamin B7 (D-Biotin) is evident both at 210 nm and 247 nm by way of several-fold increase in absorbance at the respective wavelengths, without some shift in RTs at 210 nm (Fig. 4).

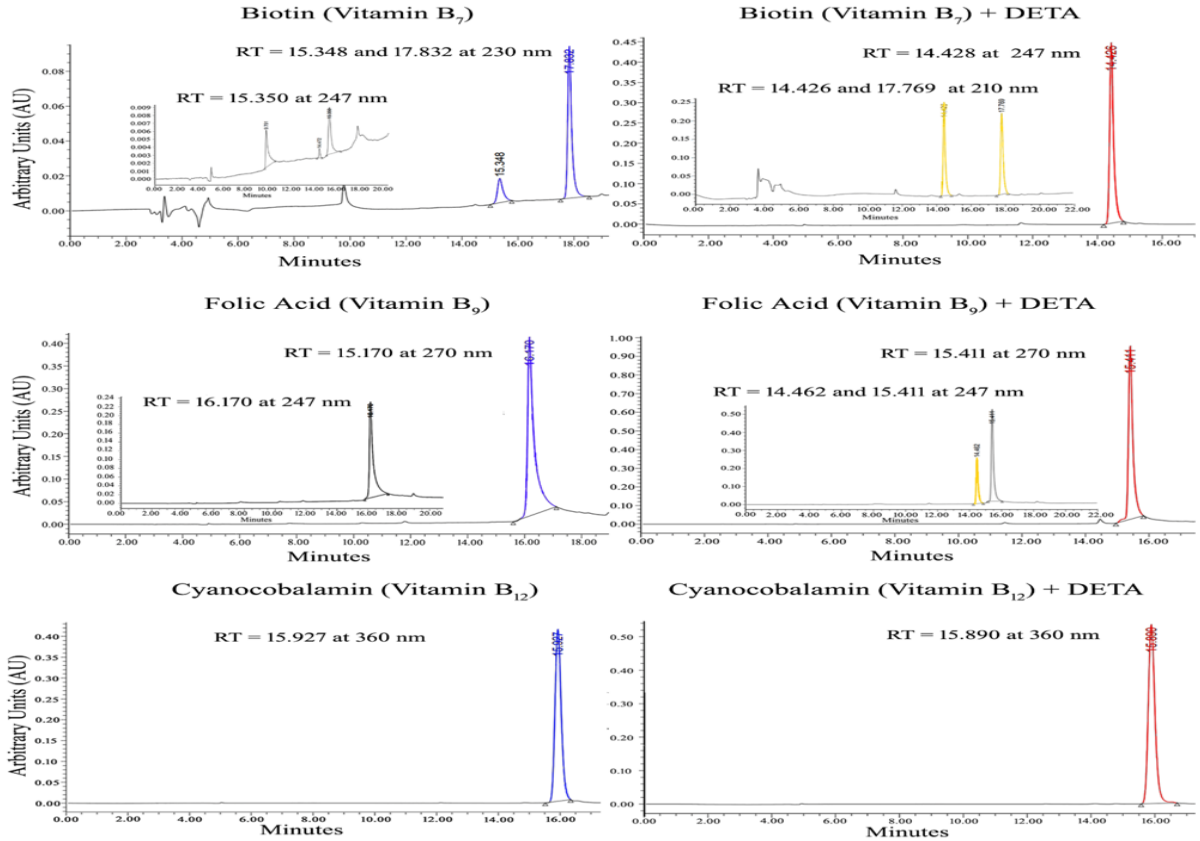


Fig. 4 HPLC analysis of D-Biotin (Vitamin B_9) and Cyanocobalamin (Vitamin B_{12}), in the absence as well as presence of NO donor, DETA, exhibit significant shifts in the retention time (RT in minutes) after incubation with DETA.

Thus, nitric oxide binding is evident. During RP-HPLC pure vitamin B9 (Folic acid) is detectable at 247 and 270 nm at an RT of 16.170. Upon incubation with DETA (NO donor), however, two sharp peaks are detected at 247 nm at RTs different from pure compound (Table V). At 270 nm, the RT remains unaltered but there is a sharp increase in absorbance. These observations indicate new product formation upon interaction of nitric oxide with Folic acid.

Table V Impact of co-incubation of NO donor (DETA) with various water-soluble vitamins (individually), as evident from HPLC analysis.

S.No.	Vitamin		Wavelength (nm)	Retention time (min)	Peak Area (%)	Absorbance (AU)	Fold-change due to NO
1.	D-Biotin (B_7)	Peak 1					
		Control	210	15.348	18.09	0.02	-
				17.832	81.91	0.09	-
		(+) NO	210	14.426	49.54	0.25 ↑	+12.5
				17.769	50.46	0.22 ↑	+2.4
		Peak 2					
		Control	247	9.791	42.90	0.006	-
				14.472	4.95	0.004	-
				15.350	52.14	0.009	-
		(+) NO	247	14.426	100	0.45 ↑↑↑	+112.5
2.	Folic acid (B_9)	Peak 1					
		Control		16.170	100	0.23	-
		(+) NO	247	14.462	29.84	0.25	-
				15.411	70.16	0.54 ↑	+ 2.35
		Peak 2					
		Control	270	16.170	100	0.42	
		(+) NO		15.41	100	0.96	
3.	Cyanocobalamin (B_{12})	Peak 1					
		Control	360	15.927	100	0.42	-
		(+) NO		15.890	100	0.54 ↑	+1.28
4.	L-Ascorbic acid (Vitamin C)	Peak 1					
		Control	247	4.187	100	1.00	-
		(+) NO	247	4.455	83.86	1.15	-
				14.479	16.14	0.55	-1.81
		Peak 2					
		Control	270	4.188	100	0.15	-
		(+) NO	270	4.455	90.59	0.18	-
				14.479	9.41	0.05	-

In case of Cyanocobalamin (Vitamin B_{12}) some increase in absorbance at 360 nm is observed without any major shift in RT (Table V). This indicates a possible interaction with NO. NO interaction with vitamin C (L-Ascorbic acid) results in the formation of two new peaks at different RTs, as is evident from UV detection at 247 nm (Table V). Likewise, at 270 nm, two new peaks are evident at RTs different from that of control. NO binding is, thus, evident (Fig. 5).

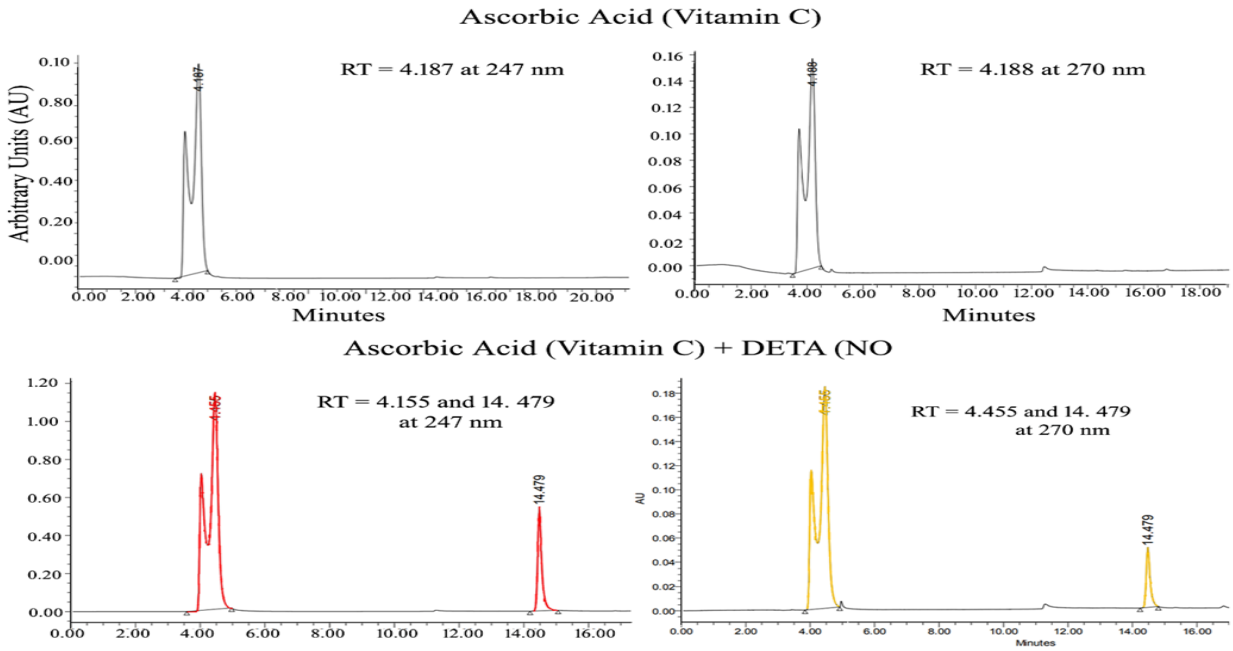


Fig. 5 HPLC analyses of 100 µg/ml of vitamin C (L-Ascorbic acid) incubated with equimolar DETA (NO donor)

IV. DISCUSSION

Separation of water-soluble vitamins of B series by sensitive techniques has been undertaken from time to time from various foods, biological fluids, beverages and pharmaceutical preparations/products [13,14,15]. Among the various methods developed over the last two decades for precise detection and quantification of water-soluble vitamins, liquid chromatography (LC) appears promising because of significant improvements in stationary phases and instrumentation [16]. Water soluble vitamins are often determined by RP-HPLC using an aqueous mobile phase whereas lipid soluble vitamins are determined using organic solvent as mobile phase both in reverse phase and normal HPLC. Both water- and fat-soluble vitamins also exhibit differences in their reverse phase retention properties. Recently, separation of eight water soluble vitamins was achieved by LC analysis within 30 min and detection at 280 nm. The use of HPLC coupled with UV detection has been reported to be simple, fast and reliable method for detection of water-soluble vitamins in food [16,17,18]. Vitamins exhibit different spectroscopic, chromatographic and voltametric properties. Errors can occur in their characterization since some of the vitamins are prone to atmospheric oxidation in solution (particularly vitamins A, D, E, K and C), to light ((vitamins A, D, E, K, B1, B2, B6 and C), to active adsorbents (vitamins A, D and K), to heat and to solvents (vitamin D) [2]. It is advisable to conduct vitamin analysis at a room temperature of 20-23˚ C, in subdued light, covering the windows with transparent UVEX foil which absorbs short wavelength actinic light [1]. Water soluble vitamins have earlier been reported to be much more stable than fat soluble vitamins on dry silica gel layers. Commonly used buffers for the separation of water-soluble vitamins are acetic acid, phosphate and formic acid. Analysis of a mixture of water and/or lipid soluble vitamins poses challenges due to differences in their solubilities. Additionally, in some cases, different biologically equivalent compounds are listed as a single vitamin. For example, niacin is available as nicotinamide and nicotinic acid. Both of them are biologically active and are referred as niacin.

Separation of various analytes by HPLC under specific conditions provides specificity of their separation with time. Thus, each analyte is presented to the instrument detector at a unique retention time. Retention of different water-soluble vitamins during HPLC can be modulated by the pH of the mobile phase. Phosphate buffer is at times used to avoid baseline absorbance shift at 210 nm when using some acids (eg, acetic or formic acid) during gradient generation. This is due to changes in the proportion of these acids in the mobile phase. Each peak in a chromatogram represents the detector response for a different compound. Shoulder peaks and split peaks often result due to the presence of two closely related compounds which are not fully resolved. The detector detects each separated compound band against a background of mobile phase as peak. Factors that affect detector response include molecular structure and detection wavelength . UV is often used as a primary detector in HPLC. So, it is important to know the UV absorption features of the analyst/s.

The UV spectra of various water-soluble vitamins being resolved by RP-HPLC (as also those of fat-soluble vitamins) significantly differ from each other due to differences in their structures. Detector response is an extremely important factor to be considered when dealing with multicomponent reaction mixtures or one or more reaction products being resolved by RP-HPLC since it is correlated with the detection of different molecular species. Multi–wavelength detection is generally recommended for achieving best sensitivity of detection. Although choosing the wavelength of maximum absorbance can be the best choice but the wavelength selected can be at times different due to interference by possible impurities (Thermo Fisher Scientific Application Note 72488). For example, although vitamin B6 has more absorption at 210 nm, impurities are likely to interfere with its detection at this wavelength. Therefore, vitamin B_6 is best detected at 280 nm where interference from impurities is eliminated (Fig. 3). Likewise, vitamin C exhibits maximum absorption at 245 nm but its detection is, at times preferred at 254 or around 265 nm (Fig. 5). These wavelengths for detection of vitamins will deviate slightly from the recommended wavelengths, depending on the solvents used for mobile phase and traces of impurities, if any. Table II lists some of the detection wavelengths reported to have been used earlier and those employed in the present work for the UV detection of various water-soluble vitamins.

The impact of nitric oxide on various water-soluble vitamins observed in the present work can be summarised as follows: 1. No change in RT or peak of absorbance (vitamin B_2 and B_5); 2. Shift in RT (Vitamins B_1 and C); 3. Increase in absorbance and shift in RT (Vitamin B_3, B_7, B_6 and B_9); and, 4. Increase in absorbance at the same RT (Vitamin B_{12}). It is thus evident that most of the vitamins of B series (except B_2 and B_5) as also vitamin C tend to respond differently in presence of NO donor, thereby indicating a possible interaction. In this context it may be noted that vitamin B_{12} (cyanocobalamin) and vitamin C (L-ascorbic acid) have earlier been reported to interact with NO. L-Ascorbic acid (reduced) has been reported to react with nitrosating species like NO^+, N_2O_3 and S-nitrosothiols. It, however, does not react directly with NO. Consequently, NO is released AsA is converted to dehydroascorbate (DHA). DHA spontaneously decays to ascorbyl radical, which can combine with NO to yield O-nitrosoascorbate [19]. The latter undergoes hydrolysis to ascorbate and NO2. As A can also scavenge $ONOO^-$ with rather slow kinetics at neutral pH but rapid kinetics at pH 5.8 yielding NO_2^- and NO_3^- via unknown intermediates. Another known scavenger of $ONOO^-$ includes vitamin E (gamma tocopherol)**.**

Drawing an analogy from nitrosyl-heme, it is further postulated that NO reacts with various oxidation states of cobalt in two major types of vitamins B_{12} -dependent enzymatic reactions [20]:

Methyltransferases Co (III) → Co (I)

Mutases Co (III) → Co (II)

Cyanocobalamine (CN-Cbl) has a cobalt atom coordinated to four nitrogen in a corrin ring. Brower et al. [21] demonstrated that NO has a high affinity for cobalamin (Cbl) in its 2+ oxidation state [Cbl(II)] at all pH conditions of the solution. Cbl (III), however, does not react with NO at neutral pH. At low pH, Cbl(III) reacts with NO in a two- step process that also reduces Cbl (III) to Cbl(II). Likewise, carbinamide, a late precursor in the biosynthesis of cobalamin (Cbl), exhibits 100 times greater affinity for NO than Cbl (II). The high affinity of Cbl (II) for NO indicates it as an efficient NO trap which may have significant biomedical applications. The Cbl (III)-NO complex is very stable but can transfer its NO moiety to haemoglobin (Hb) and glutathione. This is accompanied by a reduction of Cbl (III) to Cbl (II), indicating that NO^+ (nitrosonium) is the leaving group [21]

To sum up, the present work provides evidence for possible interaction of water-soluble vitamins (except B_2 and B_5) with nitric oxide. The evidence from RP-HPLC will be further authenticated by NMR analysis to elucidate the expected molecular interactions. These findings carry important physiological significance since they will provide new reports on the mechanisms of antioxidative/ROS scavenging roles of water-soluble vitamins, facilitated by their interaction with nitric oxide.

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STATEMENT AND DECLARATION

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