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HPLC/MS Analysis of Riboflavin Sodium Phosphate Consisting of Several Structural Isomers Together with Thiamine HCL, Pyridoxine HCL, Dexpanthenol, and Nicotinamide in A Multi-Vitamin Injection Solution

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Abstract **Original Research Article**

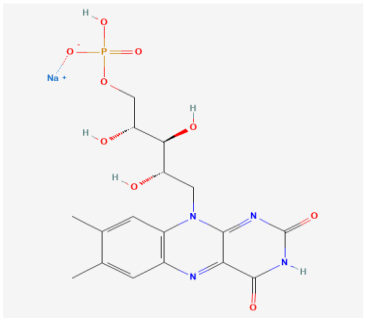
We were tasked with testing a multicomponent aqueous solution for injection used in medicine. The composition of the solution included the following components: Riboflavin sodium phosphate (vitamin B_2), Thiamine HCl (vitamin $B_{1)}$, Pyridoxine HCl (vitamin B), Dexpanthenol (vitamin B5), and Nicotinamide (vitamin B3), also benzyl alcohol. The complexity of the simultaneous determination of these components was that they differ in their acid-base properties, physical-chemical properties, concentration in solution, and solubility in the mobile phase. The key to solving this task is the correct choice of method for analyzing riboflavin sodium phosphate in each vitamin mixture. Riboflavin is taking particular attention because this component has a few isomers that are separated by HPLC.

Keywords: Riboflavin sodium phosphate, structural isomers, Pyridoxine HCl, Dexpanthenol, and Nicotinamide, thiamine hydrochloride, HPLC/MS; RP-C18, method validation.

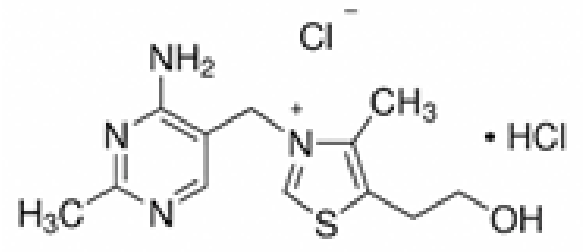
INTRODUCTION

The Aims of the work are: Find a method for quantitative HPLC/MS determination of total Riboflavin sodium phosphate, consisting of several structural isomers. Develop and validate an HPLC/MS method for the simultaneous determination of Riboflavin sodium phosphate (vitamin B2), Thiamine HCl (vitamin B1), Pyridoxine HCl (vitamin B), Dexpanthenol (vitamin B5), and Nicotinamide (vitamin B3) in an injection solution. Validate the method for accuracy, precision, repeatability, selectivity, specificity, and robustness according to (ICH Q2 (R2) 2023). Riboflavin sodium phosphate $C_{17}H_{20}N_4NaO_9P$ (Riboflavin 5'-phosphate sodium or FMN-Na) is the sodium salt of the flavin mononucleotide (FMN), or riboflavin-5′-phosphate. It is a biomolecule produced from riboflavin (vitamin B_2) by the enzyme riboflavin kinase and functions as the prosthetic group of various oxidoreductases, including NADH dehydrogenase, as well as cofactor in biological blue-light photoreceptors. Phosphorylation of riboflavin yields a complex mixture of various riboflavin phosphates (Nelsen P. *et al.*, 1986). A variety of isomeric riboflavin monophosphates can be separated by reverse-phase HPLC (Nelsen P. *et al.*, 1986). The HPLC chromatogram of a standard solution of sodium riboflavin phosphate has a few peaks (Ramesh B., 2016). Thiamine HCl (thiamine hydrochloride). "Thiamine (vitamin B_1) is used as a dietary supplement when the amount of thiamine in the diet is not enough" (Medical Encyclopedia, 2022). Thiamine hydrochloride is *soluble* in water (50 mg/ml), in *ethanol* (1 g/100 ml), in absolute *ethanol* (1 g/315 ml), and insoluble in *ether*, benzene, *hexane*, and *chloroform*. It is stable at acidic pH but is unstable in alkaline solutions (PubChem). The thesis of Tang is devoted to the HPLC analysis of various water-soluble vitamins in pharmaceutical preparations including thiamine. The paper discusses the advantages and disadvantages of various RP columns and the composition of the mobile phase (Trang H., 2013). The work of Sánchez-Machado is devoted to the simultaneous determination of thiamine and riboflavin in seaweeds by HPLC. The authors used a fluorescent detector; the mobile phase was a mixture of acetate buffer and methanol (Sánchez-Machado I. *et al*., 2004). Analysis of vitamin B1 using pre-column and post-column derivatization is described in the works of H. Ihara and M. Ofitserova, respectively (Ihara H., *et al.*,

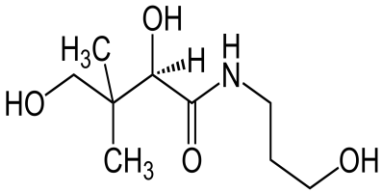
2001; Ofitserova M., *et al.*, 2013). In both studies, an HPLC instrument with a fluorescent detector was used, and the mobile phase was based on phosphate buffer and acetonitrile. Pyridoxine HCl (*vitamin B6)*. $C_8H_{12}ClNO_3$ 205.64 g/mol, 1g dissolves in 4.5 ml of water (PubChem). Pyridoxine hydrochloride is the hydrochloride salt of pyridoxine. Pyridoxine is in the vitamin B family of vitamins. Using HPLC for analysis of the Pyridoxine HCl is described in the publication (Bartzatt R., *et al.*, 2019). Dexpanthenol (D-panthenol) $C_9H_{19}NO_4$, 205.25 g·mol^{-1}, Highly viscous, colourless liquid. dexpanthenol is the alcohol analog of pantothenic acid (vitamin B_5) and is thus a provitamin of B_5. Using HPLC for analysis of the dexpanthenol is described in the publication (Mahboubi A. *et al*, 2019). Nicotinamide is a form of vitamin B_3 found in food and used as a dietary supplement and medication. Aura Industries offers a protocol for the determination of nicotinic acid and nicotinamide by HPLC with a fluorescent detector and post-column chemical derivatization (Hillebrand E. *et al.*, 2024). Nicotinic acid and nicotinamide adenine nucleotide were determined by HPLC with UV detector and LC/MS/MS methods (Yoshino J., *et al.*, 2013). The joint determination of nicotinamide and thiamine in foods is described in (Anyakora C. *et al.*, 2008). Nicotine amide in dietary supplements was analyzed by thin-layer chromatography followed by HPLC/MS analysis of the extract (Neamţu T. *et al.*, 2020). The molecular structures of the test substances and their important characteristics are shown in Figure 1.



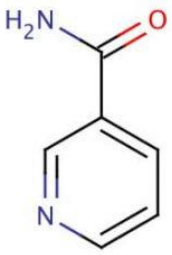
Riboflavin 5'-phosphate sodium. MW: 478.3 g·mol^{-1}, pKa1=8.26 (25°C), Sol.= 2.21 mg/mL at 25 °C



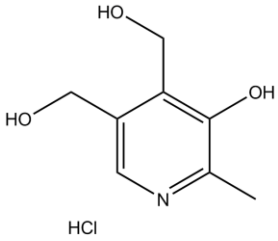
Thiamine hydrochloride. MW: 337.3g·mol−1, pKa = 4.8 (20°C), Sol.=1g in 1ml (PubChem)



Dexpanthenol. $C_9H_{19}NO_4$, MW: **205.254** g·mol^{-1}, miscible with water.



Nicotinamide. $C_6H_6N_2O$ MW: 122.127 g·mol^{-1}, pKa = 3.35 (conjugate acid) (25°C), Sol.= 1000 g/L at 20°C (PubChem)



Pyridoxine HCl 205.64 g/mol, Sol.=0.2 g/mL at 20°C

Figure 1: Molecular structures and characteristics of the tested substances

MATERIAL AND METHODS

Chemicals

Water HPLC grade purchased from Agilent. HPLC-grade solvents were used. Reference standards of Riboflavin 5'-phosphate, thiamine hydrochloride, Dexpanthenol, Nicotinamide, and Pyridoxine HCl were from Sigma. Vitamin B complex injection solution containing all 5 vitamins (Thiamine Hydrochloride, Riboflavin 5’ phosphate Sodium, Pyridoxine Hydrochloride, Dexpanthenol, and Nicotinamide) and also Benzil Alcohol was purchased from SandsRX Pharmacy.

Mobile Phase (MP): 0.1% Formic acid in Methanol/H2O 80/20.

Samples

All the samples were from a freshly prepared product. The drug solution is diluted with MP 1:10, filtered through the 0.45µm cellulose acetate membrane filter, and tested.

Instrument

Agilent HPLC/DAD/MS instrument consists of the following components: Diode Array Detector (DAD). The following wavelengths have been established: 266 nm (Thiamine HCl and Riboflavin sodium phosphate sodium), 260 nm (Nicotinamide), 222 nm (Pyridoxine HCl), and 192nm (Dexpanthenol). Reversed-phase (RP) Column Poroshell 120 EC-C18 250x4.6mm with particles size 2.7 µm, and guard precolumn; Quaternary pump with the flow: 0.7 ml/min, column temperature=40°C, and high-pressure limit of 600 bar. Single Quadrupole mass selective detector (MSD) with electrospray ionization and 150 V fragmentor, the gas temperature is 300°C, the capillary voltage is 4000 V, and the nebulizer is 15psi.

Qualitative analysis of the components was carried out using UV and MS spectra specific for each of the components (Fig. 3 and 4). Based on these spectra, 4 working wavelengths were chosen, namely 192, 222, 260, and 266 nm.

Quantitative analysis was done using a calibration curve built for each of the components.

The system's suitability has been validated according to the Center for Drug Evaluation and Research (CDER, 1994) and the System Suitability Assessment Guidelines (Evaluating System Suitability CE, GC, LC, and A/D ChemStation, 2019). The parameters were peak area, retention time, number of theoretical plates (N), and tailing factor (T).

Calibration curve and coefficient of correlation

The concentration range of the calibration curve was chosen so that the expected concentration of the component was near the middle. In this range, the calibration curve should be strictly linear ($r \geq 0.999$).

The precision/accuracy of the method was determined by the RSD value from the analysis of five samples of the same concentration under the same experimental conditions. The intraday and interday analysis was compared by RSD and recovery.

Limits of Detection (LOD)

LOD characterizes the sensitivity of a method; it is the minimum amount of a substance that can be measured by a given method, whereas the LOQ is the lowest concentration with acceptable linearity, accuracy, and precision. If the equation of the calibration curve is an equation of the first degree (straight line) then LOD is calculated by formula [1].

$$\mathbf{LOD = 3.3^* \sigma/a} \quad [1]$$

where (σ) is the residual standard deviation of the regression line, and (a) is the slope of the line (ICH Q2(R2) 2023) LOQ is 3 times LOD.

A measure of repeatability is the RSD of the mean of five independent tests of the samples of the same concentration.

To prove the specificity of the method, the peak areas of the component in the drug sample and the standard solution of the same concentration were compared. At the same time, the retention time of the component in both chromatograms was almost the same (RSD<1.2%). A minor discrepancy in the magnitude of the peak area indicated the specificity of the method for this component.

To demonstrate the robustness of the method flow rate, column temperature, and mobile phase composition were varied. The tailing factor (T) and the number of theoretical plates (N) were calculated. The results were compared with the acceptable limits.

Statistical analysis included calculating mean, standard deviation (S.D.), relative standard deviation (RSD), and correlation coefficient (r). Results $p < 0.05$ were considered statistically significant. The Least-squares regression analysis was used. In most cases, the calculation was performed automatically by the OpenLAB CDS program.

RESULTS (AND DISCUSSION)

As noted above, among the five water-soluble vitamins analyzed, riboflavin 5'-phosphate sodium occupies a special place due to the diversity of its isomers. The chromatogram of the riboflavin standard solution has four peaks, and the UV and MS spectra of these four peaks are identical (Fig. 4). The identity of the spectra indicates that these are structural isomers of riboflavin. The question arises of which peak to use for quantitation. The simplest way is to use the largest peak (Ramesh B., 2016), but we use a different approach. For each of the four peaks, we constructed calibration curves and selected the peak for quantitative analysis whose calibration curve had the highest correlation coefficient (r) (Table 1). For quantitation, we use peak number four.

Table 1: Characteristics of 4 isomers of riboflavin 5'-phosphate sodium

Peak #	RT (min)	r
1	4.26	1.0000
2	7.09	0.9804
3	15.74	0.9623
4	17.5	1.0000

System suitability (Table 2)**.** The standard solution of each of the components was tested five times. The results were averaged, and the RSD was calculated

automatically using the OpenLAB CDS software. The acceptable limit is in line with the recommendations (Dr. Deepak, 2013; Bose A., 2014).

Table 2: System Suitability Results for 5 vitamins, n=5

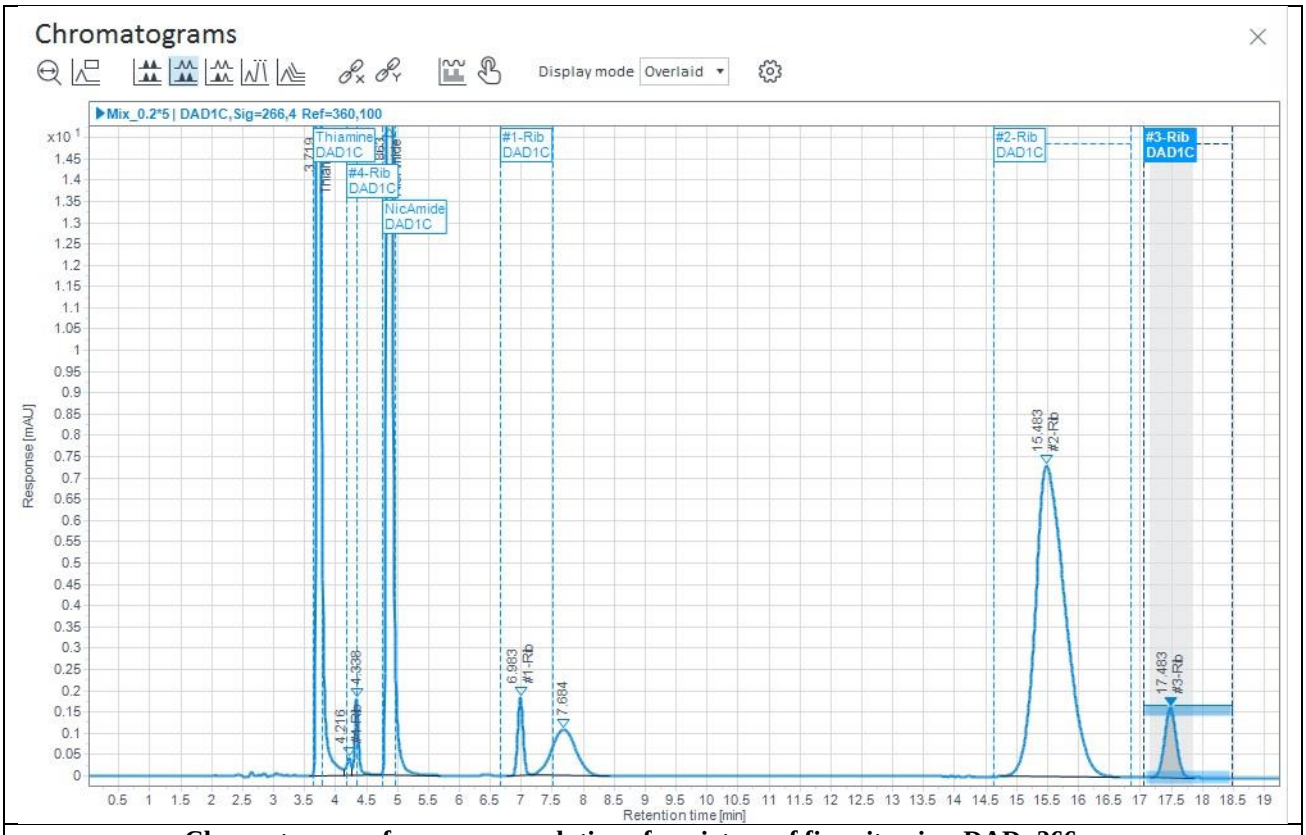
Test parameters	Acceptable limit	riboflavin 5'-phosphate sodium	Thiamine HCL	Dexpanthenol	Nicotinamide	Pyridoxine HCl
RSD of retention time	RSD ≤2	1.05	1.01	1.1	1.01	1.02
Tailing factor (T)	≤2	1.3	1.4	1	1.2	1.2
Resolution *	≥ 2	6	4	21.8	4.6	5.7
Theoretical plates (N)	>2000	106682	75439	155800	110595	105831

(*) resolution between the two closest peaks in the chromatogram of a solution of a mixture of five vitamins

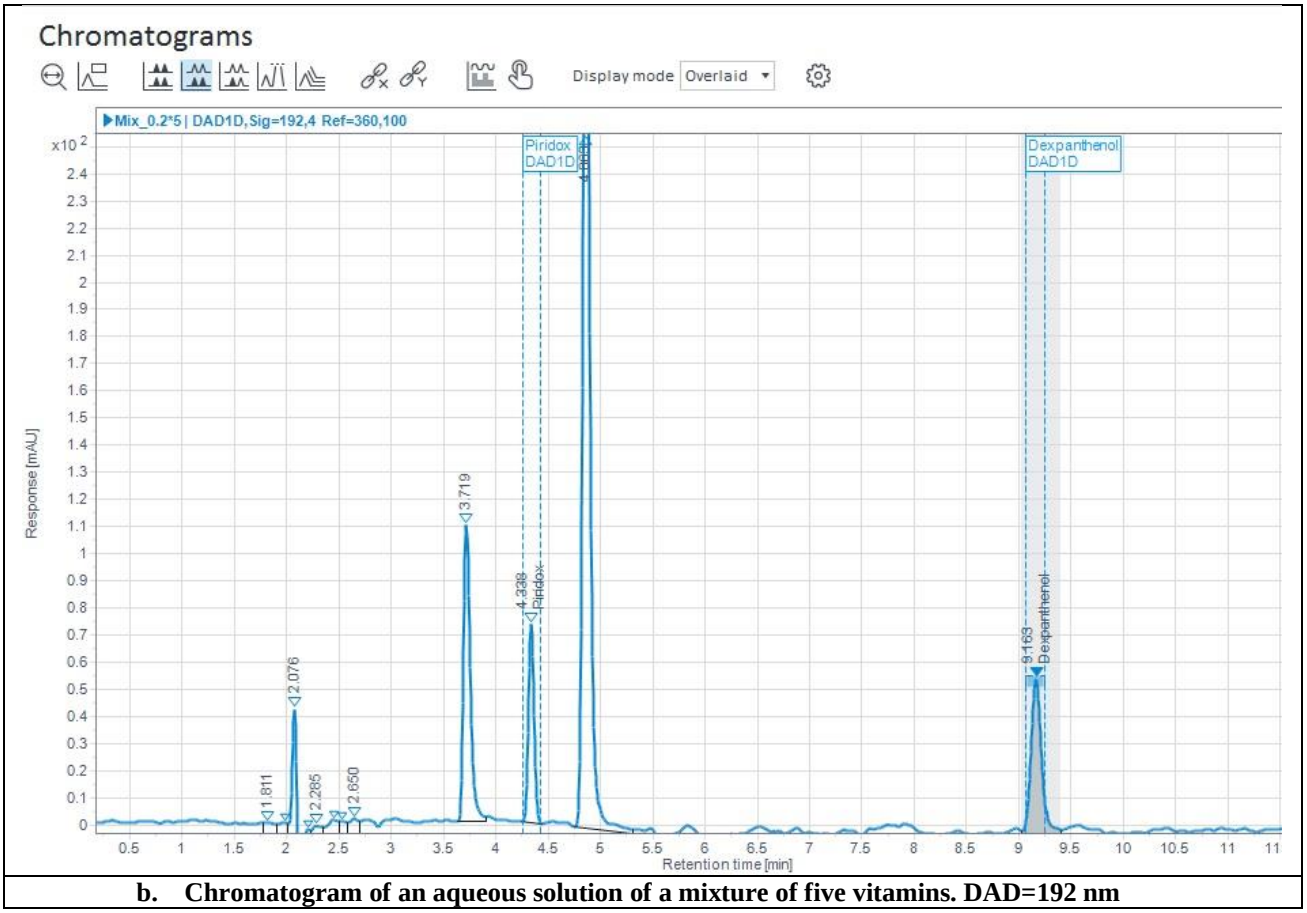
Qualitative Analysis

Qualitative determination of vitamins in solution was carried out using three parameters, namely, retention time, UV spectrum, and MS spectrum. The riboflavin chromatogram (Figure 4.) has several peaks with retention times that are constant for the given analysis conditions. The UV spectra for these peaks are identical; they have three maxima. The MS spectrum (Figure 4.) haze a main signal M/Z+=457.3 which corresponds to protonated riboflavin phosphate. The RT's and MS spectrums of 4 other components haze the following numbers: Dexpanthenol RT=9.1, m/z+=206.3 it corresponds to protonated dexpanthenol, Pyridoxine HCl RT=4.3, m/z+=170.2 corresponds to protonated pyridoxine, Nicotinamide RT=4.9, m/z+=123.2 corresponds to protonated nicotinamide, Thiamine HCl RT=3.7, m/z+=265.2 corresponds to thiamine cation.

The chromatograms are presented in Figure 2. The extracted UV and MS - spectra are presented in Figures 3 and 4.



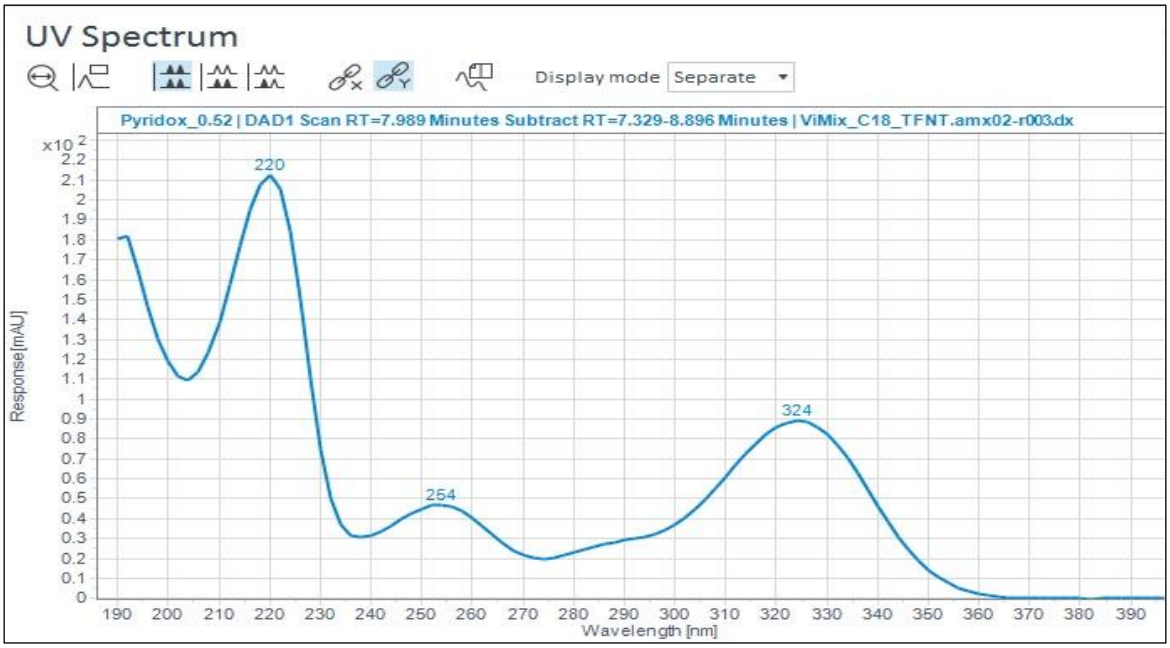
a. Chromatogram of an aqueous solution of a mixture of five vitamins. DAD=266 nm

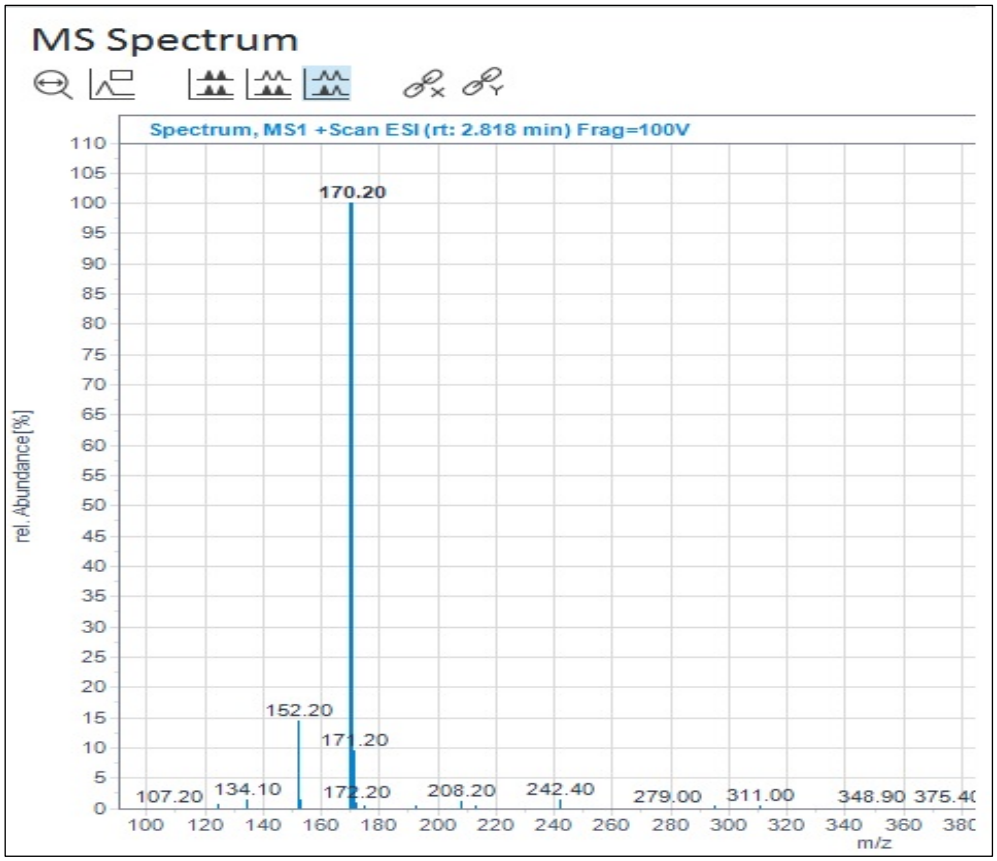


b. Chromatogram of an aqueous solution of a mixture of five vitamins. DAD=192 nm

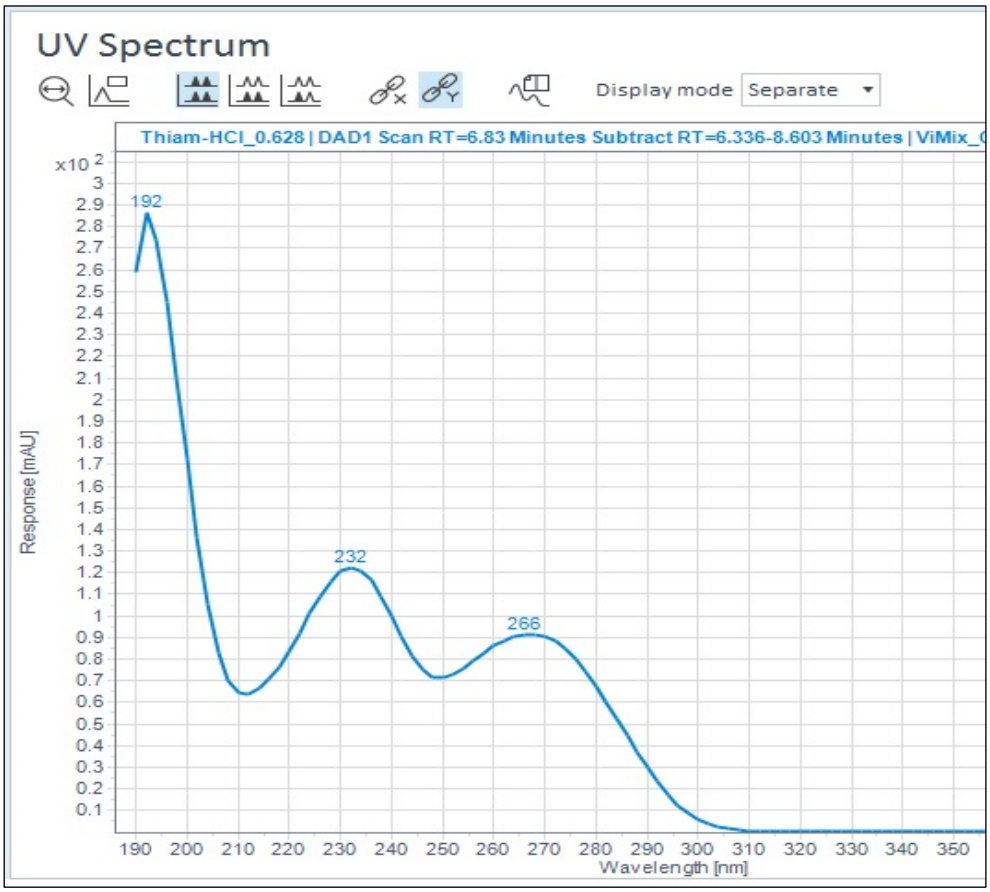
Figure 2: Chromatograms of the multivitamin injection solution.

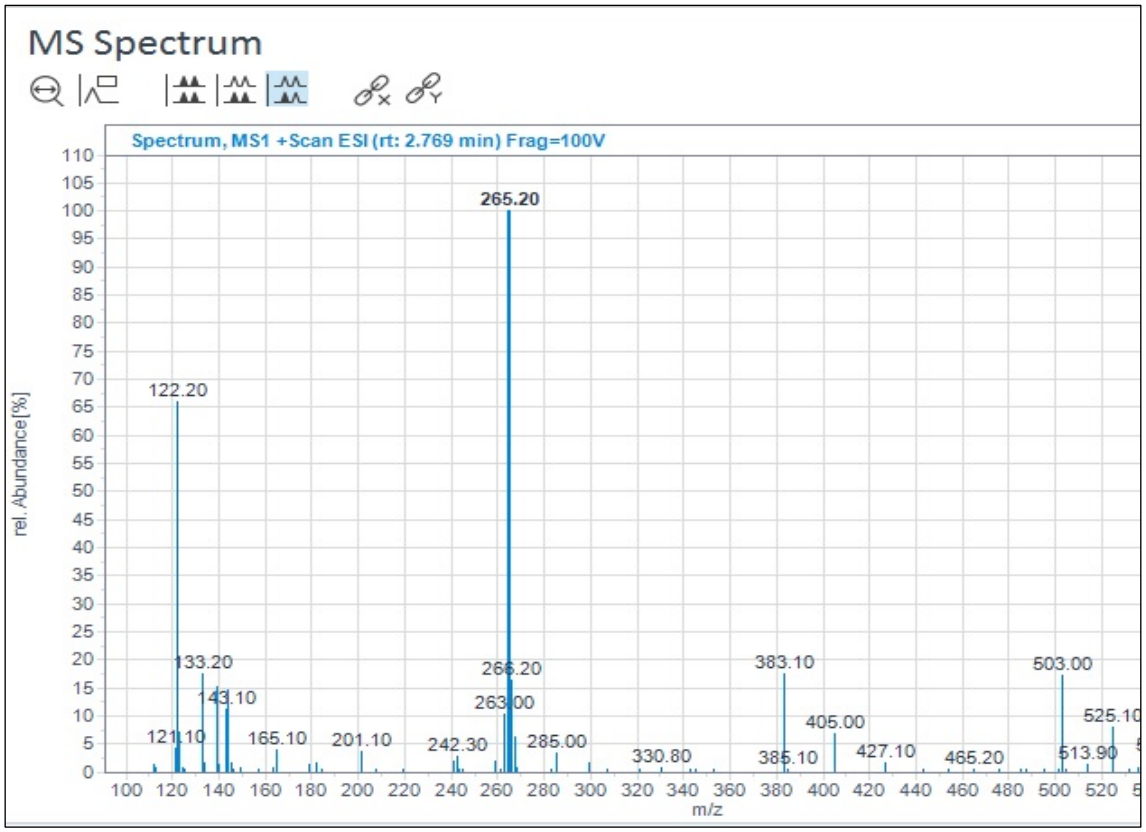
All five tested components are well separated. The slowest component, an isomer of Riboflavin 5'-phosphate sodium, has a retention time of about 18 minutes so the run time was set to 30 minutes. UV spectra of the vitamins are presented in Figure 3, 4. Each of the spectra has its specific maximums and may be used for qualitative analysis. Tryptophan has a maximum of 218 nm, niacin has maximums of 210 and 262 nm, thiamine has maximums of 232 and 266 nm, and Riboflavin 5'-phosphate sodium has a maximum of 224 and 268 nm, and Dexpanthenol – 192nm.



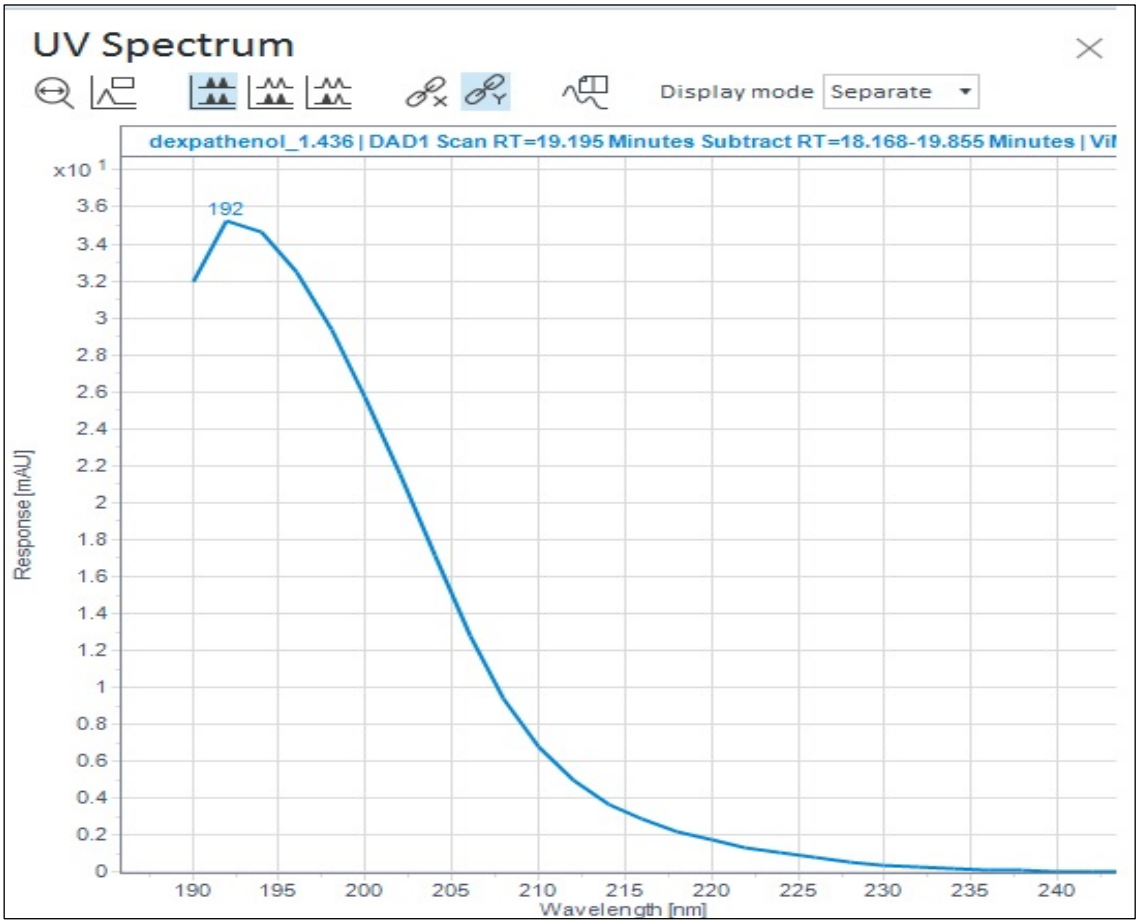


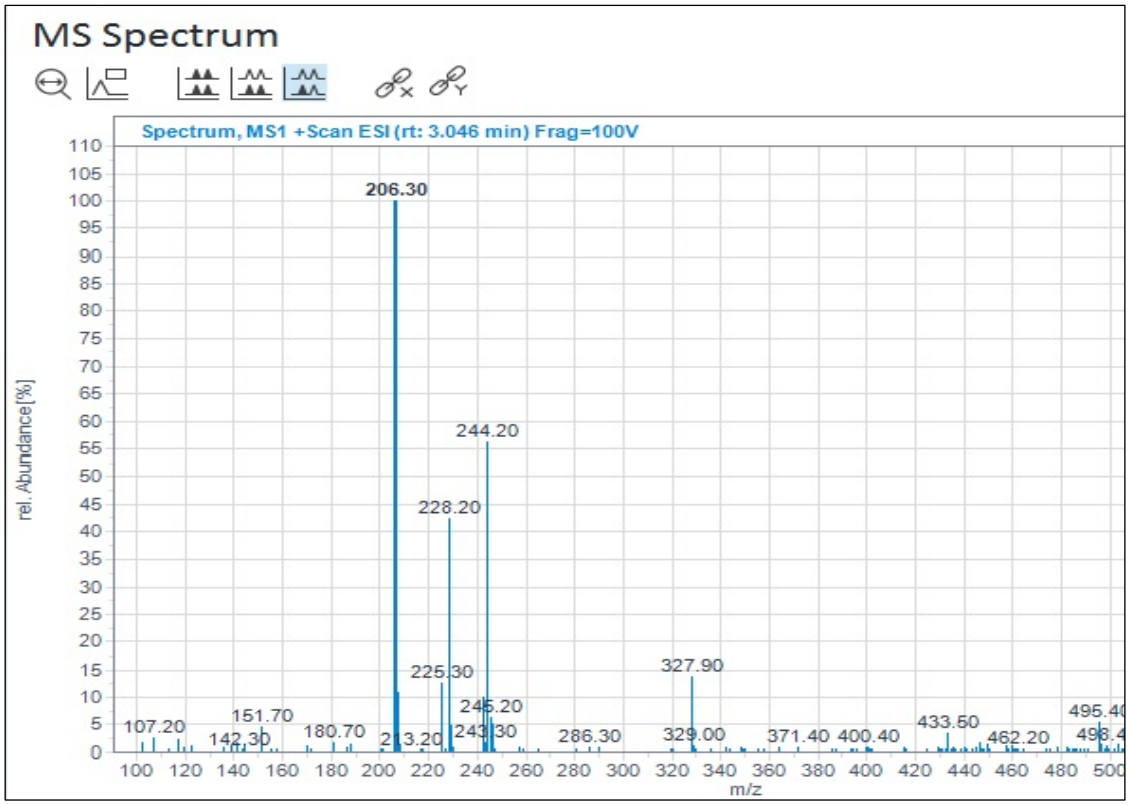
Pyridoxine HCl



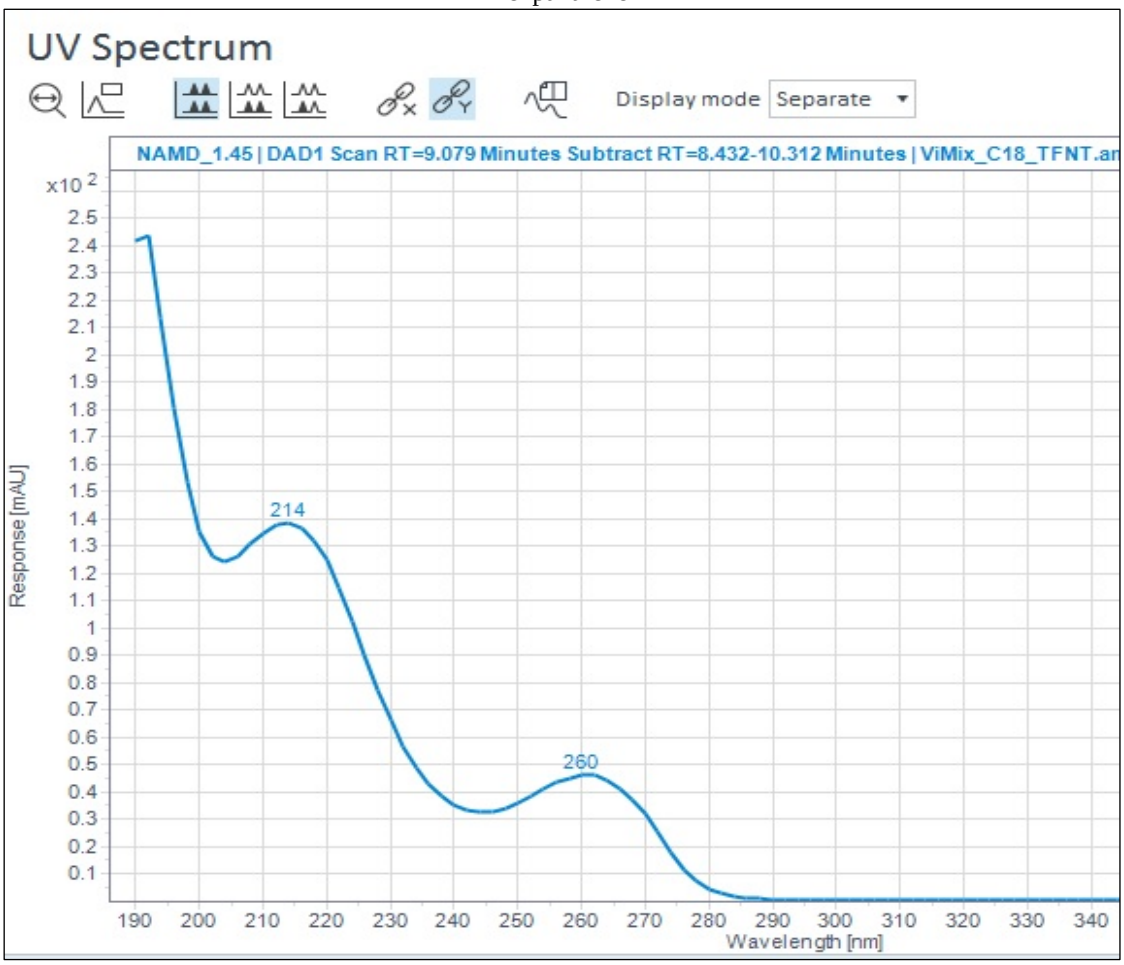


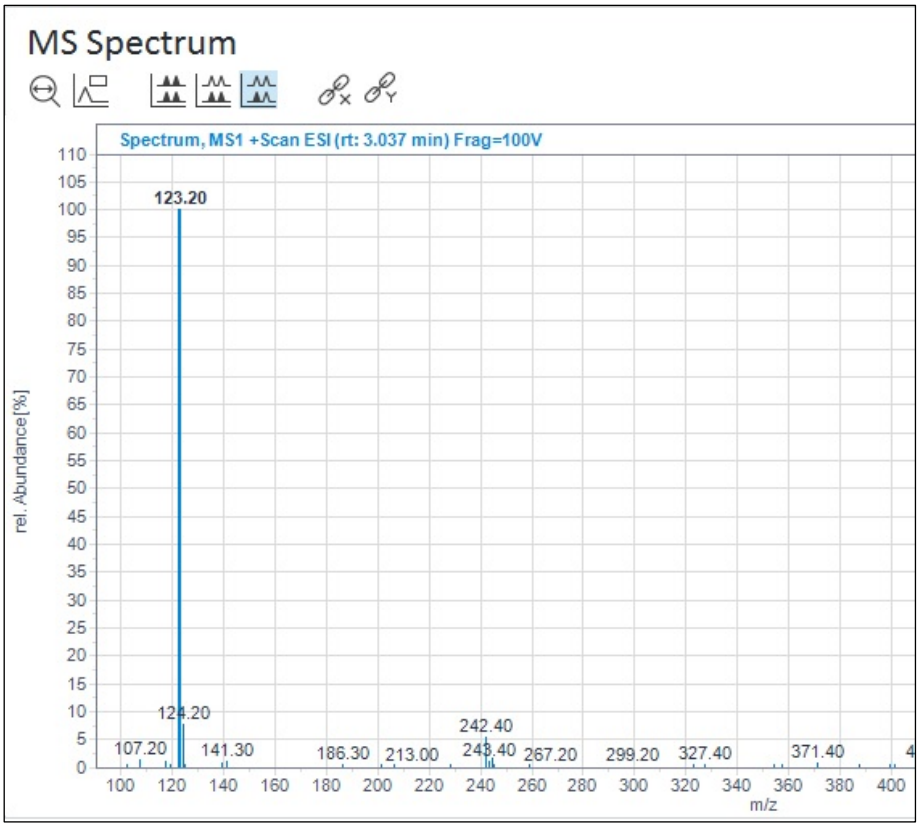
Thiamine hydrochloride





Dexpanthenol





Nicotinamide

Figure 3: Spectra of four vitamins

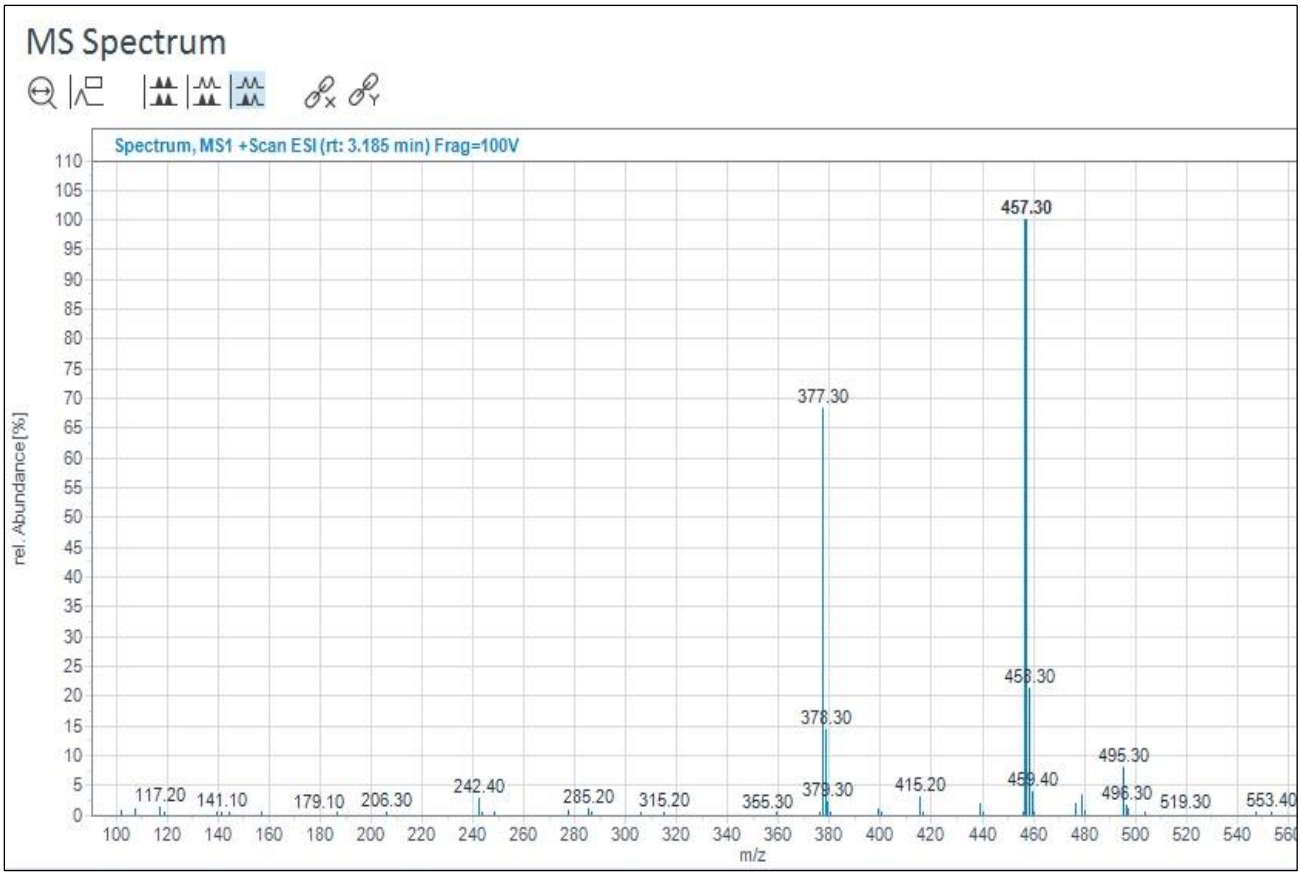


Figure 4: Riboflavin 5'-phosphate sodium standard solution. Top panel –UV Spectra of the 4 main peaks (RT= 4.26, 7.09, 15.70, and 17.70 min). Bottom panel – MS spectra

Linearity, Range, and Limit of Detection

The working range for each of the four components is set, it corresponds to the range of the linear section of the calibration curve (Table 3). The linearity is more than satisfactory, the correlation coefficient is almost equal to one. The limit of detection is more than satisfactory for testing pharmaceutical products (Table 3). We state the LOD in micrograms, which is complete information on the sensitivity of the method, as opposed to indicating the minimum concentration, as some authors do, since indicating the concentration without specifying the injection volume creates uncertainty. The typical injection volume of our device is between 0.1 and 20 microliters.

Table 3: Calculation of LOD of 5 vitamins

Riboflavin 5-phosphate-Na		**Dexpanthenol**		**Nicotinamide**		**Piridoxin HCl**		**Thiamine HCl**	
Range (µg)	**0.01 - 1.00**	**Range (µg)**	**0.01 -1.00**	**Range (µg)**	**0.01 -20.00**	**Range (µg)**	**0.01 -1.00**	**Range (µg)**	**0.01 -10.00**
a	126	a	81134	a	1733	a	5535	a	2020
b	0.00	b	0.01	b	-0.01	b	-1.10	b	0.00
r	0.9999	r	0.9999	r	1.0000	r	1.0000	r	1.0000
σ mean	-0.02	σ mean	0.50	σ mean	0.10	σ mean	0.10	σ mean	0.50
S.D. σ	0.21	S.D. σ	6.98	S.D. σ	4.31	S.D. σ	2.92	S.D. σ	7.23
LOD (µg)	**0.0056**	**LOD (µg)**	**0.0003**	**LOD (µg)**	**0.0080**	**LOD (µg)**	**0.0017**	**LOD (µg)**	**0.0120**

Accuracy/recovery and precision (Table 4). Samples containing three different concentrations of the component of interest were measured five times, and the mean value and the relative standard deviation were calculated. The recovery was determined based on the calibration curve. The data in Table 4 confirm the accuracy, reproducibility, and precision of the method. Interday analysis (check the next day) shows no significant degradation.

Table 4: Accuracy/recovery and precision

Riboflavin 5-phosphate-Na (µg)	Mean recovery (µg)	S.D.	RSD (%)	Recovery (%)
0.3	0.290	0.003	1.00	97
0.8	0.810	0.008	1.00	101
1.2	1.190	0.012	1.00	99
0.8	0.79	0.008	1.00	99
Dexpanthenol (µg)				
0.03	0.031	0.000	1.00	103
0.15	0.149	0.002	1.00	99
0.22	0.220	0.002	1.00	100
0.15	0.148	0.002	1.00	99
Nicotinamide (µg)				
2	1.98	0.020	1.00	99
5	5.1	0.050	1.00	102
10.3	10.2	0.103	1.00	99
5	4.9	0.050	1.00	98
Pyridoxin HCl (µg)				
0.02	0.019	0.004	1.00	95
0.05	0.052	0.004	0.00	104
0.19	0.196	0.008	0.00	103
0.05	0.048	0.004	1.00	96
Thiamine HCl (µg)				
0.2	0.201	0.002	1.00	101
0.5	0.498	0.005	0.00	100
0.81	0.8	0.008	0.00	99
0.5	0.501	0.005	1.00	100

Recovery data presents an average value of five independent determinations (n=5). The highlighted line corresponds to the inter-day analysis.

Selectivity (Specificity) assay. The results of the analysis of the standard solution and the test solution with the same concentration of the test component were compared. The presence of other ingredients does not affect the recovery of the tested component. The relative standard deviation of the compared peak areas does not exceed 1.5% (Table 5). Thus, the method is specific to each of the tested components.

Table 5: Selectivity (Specificity)

Active component	µg per injection	Mean peak area. (Standard) (n=5)	Mean peak area (Drug) (n=5)	RSD (%)	MS spectrum of the peaks (m/z+)
Riboflavin 5-phosphate Na	0.16	20.25	20.01	0.84	257.3
Dexpanthenol	0.033	321	320	0.24	206.3
Nicotinamide	0.43	2108	2121	0.43	123.2
Pyridoxin HCl	0.52	326	324	0.43	170.2
Thiamine HCl	1.44	35024	35724	1.4	265.2

Two peaks are compared, one for the standard solution and the other for the diluted injection solution. The concentration of the test component in both solutions is the same.

Robustness (Table 6). As part of establishing the robustness of the method, the chromatographic parameters (T and N) of each of the five components were determined with a change in flow rate, column temperature, and composition of the mobile phase. These parameters changed insignificantly and were within acceptable limits. Thus, the method is robust.

Table 6: Robustness

Parameter	Riboflavin 5'-phosphate sodium. 0.16μg		Dexpanthenol. 0.22μg		Pyridoxine HCl. 0.102μg		Nicotinamide. 0.29μg		Thiamine HCl 0.126μg	
	T	N	T	N	T	N	T	N	T	N
Flow rate 0.70 mL/min	**1.3**	106682	**1.0**	21829	**1.2**	26161	**1.2**	27649	**1.4**	18860
Flow rate 0.75 mL/min	1.3	102688	1.000	22002	1.2	24142	1.2	27649	1.7	19213
Temperature 38°C	1.3	105221	1.000	21990	1.2	25139	1.2	27649	1.8	17552
Temperature 40°C	1.3	106682	1.000	21829	1.2	26161	1.2	27649	1.7	18860
Mobile phase composition										
Formic acid 0.1%	1.3	106682	1.000	21829	1.2	26161	1.2	27649	1.7	18860
Formic acid 0.13%	1.3	152382	1.000	21600	1.2	27005	1.2	27649	1.7	18555
T= Tailing factor (mean); N= number of theoretical plates (mean); n=5.										

CONCLUSION

A fundamental point in this work is the choice of standard for constructing the Riboflavin 5'-phosphate sodium calibration curve. The standard must be of the same origin as the component of the vitamin mixture. Otherwise, the distribution between isomers may be different. We recommend that analysts request a sample of the Riboflavin 5'-phosphate sodium they are using to prepare the mixture from the client and use this sample as a secondary standard. The method makes it possible to analyze all five components simultaneously without derivation and special pretreatment of the sample. The method has high sensitivity, selectivity, specificity, and robustness. The method can be recommended for the analysis of all five components, any part of them, or each of the components separately. The method we have developed for constructing a calibration curve for riboflavin sodium phosphate can be generalized and recommended for analyzing the total content of a substance consisting of a mixture of isomers.

Conflict of Interest: The authors claim that there is no conflict of interest.

Abbreviations:
DAD-Diode Array Detector
FDA-Food and Drug Administration
ICH-International Conference on Harmonization
LOD–limit of detection
MSD-mass selective detector
MP-mobile phase
MW-Molecular weight
N–Number of theoretical plates
RP-reversed-phase
RSD–relative standard deviation
SQ-single quadrupole
T–Tailing factor
UV-VIS-Ultraviolet-Visible
Sol-solubility in water

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