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A novel RP-HPLC method for simultaneous determination of vitamins B_1, B_2, B_3, B_6 and C in oral powder for veterinary consumption

Ahmet HAŞİMOĞLU [1]*, Shantanu B. GHODKE [1]

[1] Research and Development, Deva Holding A.Ş., Çerkezköy-2, Atatürk Mh. Fatih Blv. No: 26 Kapaklı, Tekirdağ, Turkey.

* Corresponding Author. E-mail: ahasimoglu@deva.com.tr, a.hmet@yahoo.com (A.H.); Tel. +90 532 645 26 69; ORCID No: 0000-0002-2526-6900.

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ABSTRACT: The water-soluble vitamins are gaining rapid importance not only for human health but also being widely recommended for animal health care because of theirs wide spectrum of activities ranging from deficiency, pathogenic condition to nutritional supplements. A plethora of articles are published for simultaneous evaluation of B-Complex Vitamins, but most of their methods are not selective and time consuming which involve pre-treatment of sample due to complexities involved in the placebo matrix. In this study, we propose a simple, specific, precise, accurate and linear method to separate vitamin B_1 (thiamine), vitamin B_2 (riboflavin sodium phosphate), vitamin B_3 (niacin amide), vitamin B_6 (pyridoxine) and vitamin C (ascorbic acid) by using Zorbax XDB C18 column (250 mm x 4.6 mm i.d., 5µm). The flow rate was kept as 1.3 mL/min and monitored at 270 nm with ambient temperature. Effective separations and quantification were obtained in 30 minutes with 10 µl injection volume. The current reverse phase gradient method resulted in significant resolution between interested vitamins and placebo matrix. Analytical method development, followed by analytical method validation was accomplished according to International Conference of Harmonization guidelines (ICH). Statistical evaluation of validation parameters proved that the results were highly specific, precise, accurate, robust and linear over a wide concentration range. This method has been validated and found to be applicable in routine analysis of vitamin B_1, vitamin B_2, vitamin B_3, vitamin B_6 and vitamin C in oral powder for veterinary consumption.

KEYWORDS: RP-HPLC; simultaneous determination; vitamin C, B1, B2, B3, B6; water-soluble vitamins; veterinary.

1. INTRODUCTION

The usage of multivitamin is gaining rapid importance in the veterinary formulations because of their multidimensional uses to treat various ailments ranging from deficiency to pathological conditions. Vitamins are organic compounds that are essential constituents of food required for normal growth, self maintenance and functioning of human and animal bodies. Different methods have been reported for determination of B-Complex vitamins ranging from derivative UV Spectrophotometry [1-3], reverse phase TLC [4], Electrophoresis [5] and reverse phase (RP)-HPLC. The standard methods are sometimes non-specific and time consuming, involve pre-treatment of the sample through complex chemical, physical or biological reactions to eliminate the interferences followed by individual methods for each different vitamin. From the aforesaid methods, RP-HPLC is extensively used instead of other methods because of its higher sensitivity. The existing RP-HPLC methods are for determination of multivitamin in human blood and plasma [6], capsules [7] and tablet dosage forms [8-13]. The main advantage of our study is to have a simple, specific, precise, accurate and linear reverse phase gradient HPLC method for qualitative, quantitative and simultaneous determination of vitamin B_1, vitamin B_2, vitamin B_3, vitamin B_6 and vitamin C in solid oral powder for veterinary consumption that was not reported before for these five vitamins at the same time. Validation of analytical method which had been in concordance with ICH Guidelines was successfully accomplished [14]. The determination of multivitamins in veterinary products is really challenging task due to the complexity in formulation that require a wide scope to develop and validate the method for the finished product. In the current study, pharmaceutical formulation was developed as powder formulation for which the qualitative composition was

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as follow; vitamin A, vitamin D_3, vitamin E, vitamin B_1, vitamin B_2, vitamin B_3, vitamin B_6, vitamin B_{12}, calcium d-pantothenate, vitamin C, vitamin K_3, Folic acid, D-biotin, Dl-methionine in a matrix of solid food additives.

2. RESULTS AND DUSCUSSION

2.1. Method development

A number of stability-indicating HPLC methods published before were tried in order to achieve appropriate system suitability and significant resolution between the B-Complex Vitamins but none of them have given adequate separation between interested vitamins in the matrix. To initiate method development, several mobile phase compositions ranging from pH 2 to 7 in combination with Methanol and Acetonitrile were used with a wide gradient study which resulted in unsuitable retention times with low resolution. Hence, ion pair in very low concentration was introduced in the mobile phase for effective separation. The gradient program was designed to initiate with higher buffer ratio (mobile phase A) to retain Vitamin C in the column which eluted in void volume due to its high polarity. Introduction of Acetic Acid in the mobile phase contributed immensely to attain a better peak shape with tailing factor ranging from 0.8 to 1.5. The working mobile phase with optimized gradient increased the retention time of polar vitamins with significant resolution which resulted in sharp and symmetrical peaks with less detector noise. All the vitamins were eluted before 20 minutes and injection time was decided to be 30 minutes because of the complex placebo composition. The placebo consists of vitamin A, vitamin D_3, vitamin E, vitamin K_3, vitamin B_{12}, calcium d-pantothenate, folic acid, d-biotin, dl-methionine, butyl hydroxyanisole, butyl hydroxytoluene, tartaric acid, tartrazine and significantly high quantity of Lactose. Mobile A and distilled water were used as diluents. Detection was carried out in the range of 200 nm to 800 nm using photodiode array detector and set at 270 nm where system suitability parameters and areas of analyzed vitamins were statistically significant. Each vitamin standard solution at limit concentration was injected separately in order to determine the selectivity and in mixture to check the degree of resolution. The separation pattern of the vitamins was as follow; C showed at RT – 2.3 min, B_3 showed at RT– 4.6 min, B_2 showed at RT– 7.4 min, B_6 showed at RT– 10.7 min and B_1 showed at RT– 17.5 min. Also, the final condition of gradient was set as describe in the section 4.1. The methods present in [12-13] are only capable to analyze vitamin B_1, vitamin B_6 and vitamin B_{12} at the same time. However, the proposed method's superior points are coming from its simplicity and analyzing five vitamins which are vitamin B_1, vitamin B_2, vitamin B_3, vitamin B_6 and vitamin C simultaneously in oral powder for veterinary consumption that is not present in the existing methods [3-5]. Beside this, being a stability indicating method is another superior side of choosing the suggested method.

2.2. Specificity

The presence of many vitamins, excipients like tartaric acid, antioxidants (butyl hydroxyanisole and butyl hydroxytoluene) and colouring agent (tartrazine) did not cause any interference with the vitamins of interest. Under these conditions every peak was well separated from each other and placebo matrix. So, the desired system suitability requirements were achieved (Table 1). Overlay chromatograms of blank, placebo, standard and sample solutions are given in Figure 1. Peak purity results of interested vitamins in sample injection were 1.00; 1.00; 1.00; 1.00; 1.00 for vitamin B_1, vitamin B_2, vitamin B_3, vitamin B_6 and vitamin C, respectively. Representative sample chromatogram where intended vitamins are well separated without any interaction of placebo matrix is given in Figure 2.

Table 1. The system suitability parameters for sample solution

Vitamin Name	Retention time (min)	Tailing factor	Theoretical plate count	Resolution factor *Rf*	*k'*
C	2.3	1.4	3564	3.2	0.7
B3	4.6	1.8	2802	3.2	2.4
B2	7.4	1.3	9181	8.6	4.5
B6	10.7	1.3	25859	11.5	6.9
B1	17.5	1.6	93350	27.4	12.0

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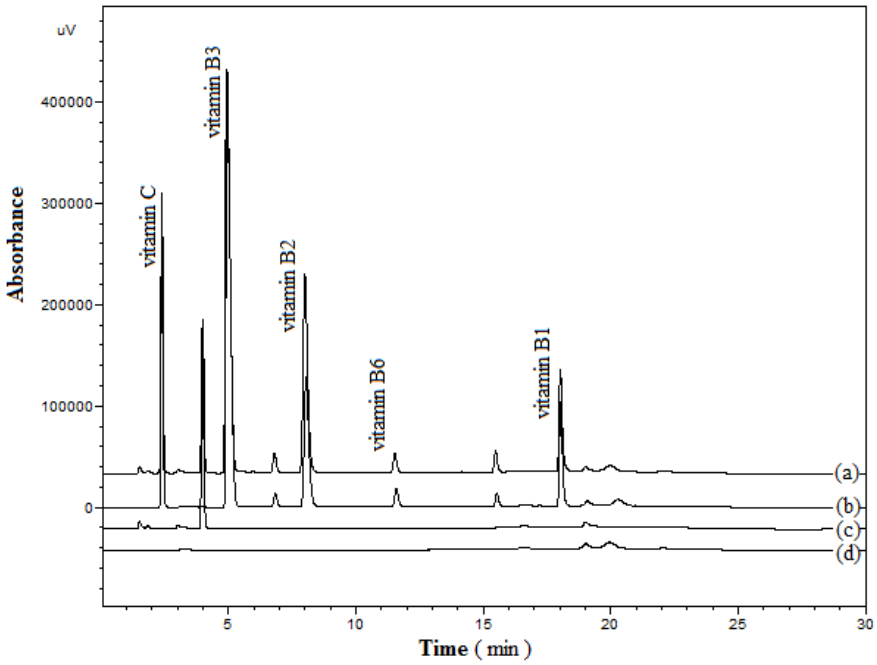

Figure 1. Comparison of (a) sample solution, (b) standard solution, (c) placebo solution and (d) blank solution chromatograms

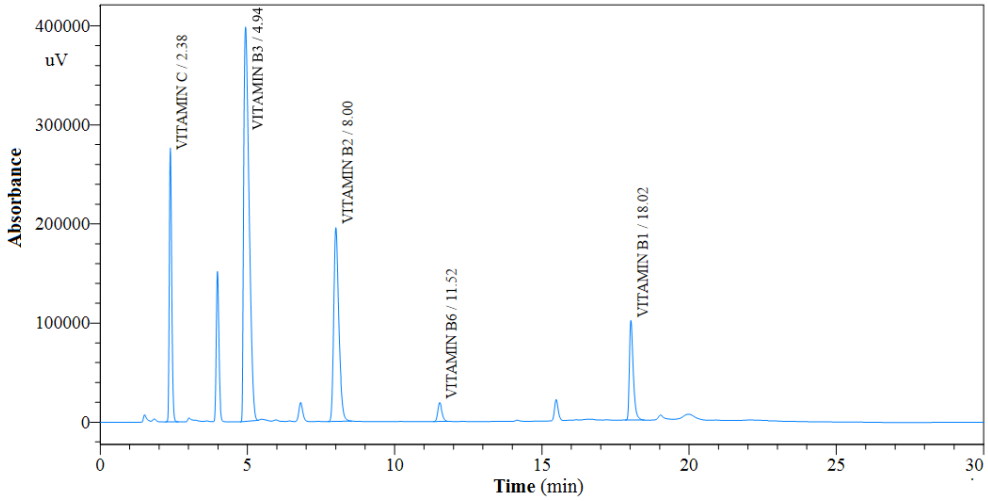

Figure 2. Sample chromatogram

2.3. Linearity

The linearity was studied at 80%, 90%, 100%, 110%, 120 % levels for each vitamin. A linearity curve was generated between area and concentration for each analyzed vitamin. Regression parameters of analyzed vitamins are given in Table 2.

Table 2. Regression parameters of analyzed vitamins

Substance	Concentration(µg mL^{-1})	Slope	Intercept	r^2
C	241.100 - 361.600	4702949.147	16088.504	0.9998
B_1	97.379 - 146.069	7621249.712	16776.162	0.9999
B_2	86.617 - 129.925	14475722.954	6939.648	1.0000
B_3	359.472 - 539.207	10960683.445	38497.162	0.9997
B_6	48.641 - 72.962	2961729.402	2620.976	0.9999

The results demonstrated satisfactory and consistent behaviour of HPLC method. Least-squares regression analysis was used to determine the data which showed excellent linearity with $R^2 \geq 0.99$.

2.4. Accuracy

Accuracy study was conducted at 3 levels ranging from 80 % to 120 %. The results of accuracy are given in Table 3.

Table 3. Representative concentrations of accuracy at 80 %, 100 % and 120 % levels

Substance	Added (µg mL^{-1})	Found (µg mL^{-1})	Recovery (%)	RSD (%)
80 % Level of Test Concentration				
C	243.826	243.338	99.80	0.07
B_1	48.401	48.063	99.30	0.06
B_2	43.832	43.948	100.27	0.10
B_3	361.382	368.146	101.87	0.06
B_6	24.785	25.026	101.00	0.06
100 % Level of Test Concentration				
C	304.783	301.423	98.93	0.06
B_1	60.501	60.162	99.47	0.06
B_2	54.790	55.184	100.70	0.10
B_3	451.728	453.364	100.37	0.06
B_6	30.982	31.424	101.43	0.06
120 % Level of Test Concentration				
C	365.739	358.771	98.10	0.10
B_1	72.601	72.598	100.00	0.26
B_2	65.748	66.066	100.47	0.71
B_3	542.074	538.206	99.30	0.10
B_6	37.178	37.615	101.20	0.26

Recoveries for all intended vitamins where minimum and maximum recovery values were in the range of 98.1% - 101.9% respectively were in-line with ICH guideline requirements. Therefore, the obtained results were satisfactory.

2.5. Precision and intermediate precision

Precision and intermediate precision analyses were conducted on two different days with two different HPLCs, columns and analysts. The low RSD % and confidence interval values were evidence of appropriate precision of this stability indicating method which is given in Table 4.

Table 4. Precision and Intermediate precision results

Vitamin Name	Precision % RSD (Mean ± Confidence Interval)	Intermediate precision % RSD (Mean ± Confidence Interval)
C	0.78 (100.6±0.9)	1.04 (99.4±1.2)
B_1	0.34 (98.4±0.4)	1.14 (98.7±1.3)
B_2	0.22 (102.1±0.3)	1.09 (101.1±1.1)
B_3	0.27 (97.9±0.3)	0.90 (101.8±0.9)
B_6	0.21 (99.0±0.4)	1.30 (100.2±1.4)

2.6. Robustness

Performed robustness study and changes due to deliberate variations are shown in Table 5. To observe the effect on proposed method, flow rate, column oven temperature, column lot number change and wavelength were changed intentionally.

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Table 5. Robustness evaluation of developed HPLC Method

Chromatographic Changes	Vitamin C			Vitamin B_1			Vitamin B2			Vitamin B_3			Vitamin B_6		
	k′	*T*	*N*	*k′*	*T*	*N*	*k′*	*T*	*N*	*k′*	*T*	*N*	*k′*	*T*	*N*
A: Temperature															
23°C	0.816	1.293	3837	11.628	1.451	86082	4.395	1.421	9107	2.388	1.432	3719	6.953	1.211	27725
25°C [a]	0.795	1.302	3928	11.136	1.402	91457	4.394	1.401	10185	2.187	1.401	4020	6.832	1.200	29109
27°C	0.724	1.310	3861	10.620	1.411	87273	3.993	1.341	9153	2.006	1.441	3998	6.451	1.220	26230
B : Flow															
1.1 mL/min	0.809	1.300	4307	11.918	1.405	84403	4.525	1.441	10525	2.489	1.442	4525	6.997	1.241	20525
1.3 mL/min [a]	0.795	1.302	3928	11.336	1.402	91457	4.394	1.401	10185	2.187	1.401	4020	6.832	1.200	29109
1.5 mL/min	0.703	1.321	3477	11.123	1.420	91209	4.192	1.403	9207	1.885	1.403	3809	6.661	1.201	24952
C : Wavelength															
268 nm	0.807	1.302	3922	11.626	1.421	88376	4.434	1.411	9754	2.349	1.462	4028	6.972	1.211	28551
270 nm [a]	0.795	1.302	3928	11.136	1.402	91457	4.394	1.401	10185	2.187	1.401	4020	6.832	1.200	29109
272 nm	0.801	1.301	3924	10.824	1.421	87054	4.268	1.410	9613	2.013	1.451	3950	6.620	1.220	28069
D :Column change															
Column 1 [a]	0.795	1.302	3928	11.136	1.402	91457	4.394	1.401	10185	2.187	1.401	4020	6.832	1.200	29109
Column 2	0.783	1.371	3604	11.621	1.421	97936	4.397	1.391	9990	2.186	1.421	3124	6.837	1.272	29301

[a] Optimum Condition

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According to the obtained data it can be stated that there is no significant effect which was observed on the system suitability parameters due to change of flow rate as ± 0.2 mL/min., ± 2°C column temperature change, using different column lot number and wavelength change from 268 nm to 272 nm. So, the method is robust since it was not affected from the above method parameters' changes. Meanwhile, it is in-line with European Pharmacopeia (Eur. Ph) [15] and United States Pharmacopeia (USP) [16] chromatographic requirements where theoretical plate number should be more than 2000, resolution between two peaks minimum 1.5 and tailing factor should be between 0.8 – 1.5 (Eur. Ph) and not more than 2.0 (USP). Therefore, the obtained results were satisfactory.

2.7. Solution stability

The solution stability was conducted for 48 hours. The standard and sample were found to be stable for 19 hours. The differences in % area for standard and sample solutions were significantly low that are shown in the Table 6.

Table 6. Results of solution stability for standard and sample solutions

Vitamin Name	Standard Solution % Area Difference (19 hours)	Sample Solution % Area Difference (19 hours)
C	0.2	1.5
B_1	0.0	0.1
B_2	0.4	0.1
B_3	0.0	0.3
B_6	0.0	0.3

3. CONCLUSION

The results obtained from the current work show that this method is specific, precise, accurate, robust and linear in concordance with ICH guidelines [14], Eur. Ph. [15] and USP [16] chromatographic requirements. The vitamins in this study are well separated from each other with significant resolution of minimum 3.2. Moreover, the vitamins in the current work do not interfere with any of the placebo matrix. The simplicity of the procedure should make it desirable for quality control analysis of vitamin B_1, vitamin B_2, vitamin B_3, vitamin B_6 and vitamin C determination in oral powder for veterinary consumption.

4. MATERIALS AND METHODS

4.1. Equipments (HPLC)

A Shimadzu LC-20AT HPLC integrated with a SPD-M20A PDA detector was used for data acquisition. Separations were achieved by using a Zorbax XDB C18 column with dimension of 250 mm x 4.6 mm and 5µm particle size. The flow rate was kept as 1.3 mL/min and monitored at 270 nm with ambient temperature. Effective separations and quantification were obtained in 30 minutes with 10 µl. injection volume. Auto sampler temperature was kept as 5°C and elution was gradient (Table 7).

4.2. Reagents and solvents

All the used reagents and solvents were analytical grade and they were used without any further purification. Methanol and Acetic acid were J.T.Baker grade with chemical purity 100.0%, 99.8%, respectively. 1-hexanesulfonic acid-sodium salt was Merck grade with chemical purity 99.6%. The used Water was HPLC grade.

4.3. Standards

All the standards used in the validation were provided by DSM Nutritional Products and were used without any further purification. Vitamin B_1, vitamin B_2, vitamin B_3, vitamin B_6 and vitamin C working standards' purities were 100.3%, 72.2%, 99.8%, 100.2% and 99.7% respectively.

4.4. Preparation of mobile phase

Mobile phase A was carefully prepared by dissolving 1.86 g 1-hexanesulfonic acid-sodium salt into 1 liter distilled water. Further, 15 ml of acetic acid was added and mixed well. Mobile phase B was prepared by

mixing methanol and mobile phase A in the ratio (90:10 V/V), passed through 0.45 µm membrane filter and degassed for 10 minutes by sonication.

Table 7. Gradient table

Time (min.)	Mobile phase A (%)	Mobile phase B (%)
0.0	97	3
0.1	97	3
0.3	85	15
2.0	85	15
4.0	80	20
7.0	77	23
12.0	65	35
14.0	55	45
17.0	45	55
20.0	40	60
23.0	70	30
24.0	90	10
25.0	97	3
30.0	97	3

4.5. Preparation of solutions

4.5.1. Preparation of vitamin C and vitamin B_3 stock standard solution

30.0 mg of vitamin C Standard and 45.0 mg of vitamin B_3 Standard were weighed into 20 mL volumetric flask. 10 mL of distilled water was added, sonicated for 2 minutes and diluted to the volume with distilled water. ($C_{Vitamin\ C}$ =1.5 mg/mL, $C_{Vitamin\ B3}$ =2.25 mg/mL)

4.5.2. Preparation of vitamin B_1, vitamin B_2 and vitamin B_6 stock standard solution

30.0 mg of vitamin B_1 Standard, 15.0 mg of vitamin B_6 Standard and 37.5 mg of vitamin B_2 Standard were weighed into 50 mL volumetric flask. 25 mL distilled water was added, sonicated for 2 minutes and diluted to the volume with distilled water. ($C_{Vitamin\ B1}$ =0.60 mg/mL, $C_{Vitamin\ B2}$ =0.75 mg/mL, $C_{Vitamin\ B6}$ =0.30 mg/mL)

4.5.3. Preparation of standard solution

4.0 mL of vitamin C and vitamin B_3 stock standard solution and 2.0 mL of vitamin B_1, vitamin B_2 and vitamin B_6 stock standard solution were transferred into 20 mL volumetric flask. It was diluted to the volume with mobile phase A and filtered through 0.45 µm RC filter. ($C_{Vitamin\ B3}$ =0.45 mg/mL, $C_{Vitamin\ C}$ =0.30 mg/mL, $C_{Vitamin\ B1}$ =0.06 mg/mL, $C_{Vitamin\ B2}$ =0.075mg/mL, $C_{Vitamin\ B6}$ =0.03 mg/mL).

4.5.4. Preparation of sample solution

2.5 g sample was weighed into the 100 mL volumetric flask. 50 mL of distilled water was added, vortexed for 2 minutes and sonicated for 2 minutes. It was diluted to the volume with distilled water. 3.0 mL was transferred into 25 mL volumetric flask. İt was diluted to the volume with mobile phase A and filtered through 0.45 µm RC filter. ($C_{Vitamin\ B3}$ =0.45 mg/mL, $C_{Vitamin\ C}$ =0.30 mg/mL, $C_{Vitamin\ B1}$ =0.06 mg/mL, $C_{Vitamin\ B2}$ =0.075mg/mL, $C_{Vitamin\ B6}$ =0.03 mg/mL)

4.5.5. Preparation of placebo solution

1.738 g placebo (without vitamin B1, vitamin B2, vitamin B3,vitamin B6 and vitamin C) was weighed into the 100 mL volumetric flask. 50 mL of distilled water was added, vortexed for 2 minutes and sonicated for 2 minutes. It was diluted to the volume with distilled water. 3.0 mL was transferred into 25 mL volumetric flask. İt was diluted to the volume with mobile phase A and filtered through 0.45 µm RC filter.

4.6. Specificity

In order to assess the separation of multivitamins from excipients, blank, placebo and sample solutions were injected to the HPLC system. Also, standard solution containing all interested vitamins and each vitamin standard solution at limit concentration were injected individually.

4.7. Linearity

Linearity was accomplished by testing the five different solutions of vitamin B_1, vitamin B_2, vitamin B_3, vitamin B_6 and vitamin C at 80%, 90%, 100% 110%, and 120% concentrations. Each of them was injected twice except 80%, 100 %, and 120 % that were injected six times. Linearity concentrations of each vitamin are given in the Table 8.

Table 8. Representative concentrations of linearity for 80%, 90%, 100 %, 110 % and 120% standard levels

	Concentration (mg mL^{-1})				
% Level	Vitamin C	Vitamin B_3	Vitamin B_1	Vitamin B_2	Vitamin B_6
80	0.2411	0.3595	0.0973	0.0867	0.0486
90	0.2712	0.4045	0.1096	0.0974	0.0547
100	0.3013	0.4494	0.1217	0.1083	0.0608
110	0.3315	0.4943	0.1339	0.1191	0.0669
120	0.3616	0.5392	0.1461	0.1299	0.0723

4.8. Accuracy

In order to evaluate the accuracy of analytical method, the study was carried out by preparing three different samples for each vitamin at 80 %, 100 % and 120 % by accepting the specification level as 100 %. Placebo mixture was added into every sample preparation.

4.9. Precision and intermediate precision

4.9.1 System precision

System precision was tested by using each vitamin peak areas of replicate six standard solution injections in precision parameter.

4.9.2 Method precision

Six different samples from finished product were prepared as indicated in sample preparation and injected twice. The assay results were calculated as %.

4.9.3 Intermediate precision

To see the effect of inter laboratory change on the method, six sample solutions were prepared by using the same batch of finished product by different two analysts, HPLCs and columns at different days. Each sample solution was injected twice.

4.10. Robustness

Standard solution was injected to evaluate method robustness with changes implemented in flow rate, column oven temperature, column lot and change of wavelength.

4.11. Solution stability

Standard and sample solutions were injected to system for 48 hours and checked for the change in % area as compared to initial standard and sample solutions.

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