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RESEARCH ARTICLE

Analytical Method Development and Validation for the Determination of Diclofenac Sodium, Vitamin B_1, Vitamin B_6 and Vitamin B_{12} in Soft Gelatin Capsule Dosage Form

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ABSTRACT

An accurate, simple, sensitive and selective reverse phase liquid chromatographic method has been developed for the determination of Diclofenac sodium, Vitamin B_1, Vitamin B_6 and Vitamin B_{12} in their pharmaceutical preparations. The determination of Diclofenac sodium was carried out on Phenomenex ODS, C-18 (150 x 4.6 mm) 5µ column using a mobile phase consisting of buffer: acetonitrile (40:60). The flow rate and run time were 1ml/min and 10 minutes respectively. The wavelength was 254 nm. The determination of Vitamin B_1 and B_6 was carried out on Inertsil ODS, C_{18} column (250 x 4.6mm;5µm) using a mobile phase consisting of a mixture of 1000mL of Sodium 1-hexane sulfonic acid solution, 730mL of water, 270mL glacial acetic acid of and 10ml of methanol. The flow rate and run time were 1.5ml/min and 10 minutes respectively. The wavelength was 284 nm. The determination of Vitamin B_{12} was carried out on Phenomenox ODS, C-18 column (150x4.6mm;5µm) using a mobile phase consisting of a mixture of water: acetonitrile (87:13) containing 0.25mL of Trifluroacetic acid. The flow rate and run time were 1.2ml/min and 10 minutes respectively. The wavelength was 361 nm. The developed method was found to be simple, specific, robust, linear, precise, and accurate for the determination of Diclofenac sodium, Vitamin B_1, Vitamin B_6 and Vitamin B_{12} in pharmaceutical formulations.

KEYWORDS

Diclofenac sodium, Vitamin B_1, Vitamin B_6 and Vitamin B_{12}, reverse phase chromatographic technique, method validation

INTRODUCTION

Diclofenac sodium is a non-steroidal anti-inflammatory analgesic[1]. The primary mechanism responsible for its anti-inflammatory, antipyretic, and analgesic action is thought to be inhibition of prostaglandin Synthesis by inhibition of cyclooxygenase (COX)[2]. It also appears to exhibit bacteriostatic activity by inhibiting bacterial DNA synthesis. It's chemical name is 2-[(2,6-Dichlorophenyl)amino]benzene acetic acid sodium salt. The molecular formula is $C_{14}H_{10}Cl_2NNaO_2$ and molecular weight is 318.13[3].

Vitamin B_1, also known as Thiamine hydrochloride is a water soluble vitamin[4]. Vitamin B_1 combines with Adenosine Triphosphate (ATP) to form thiamine

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pyrophosphate, also known as Co-carboxylase, a coenzyme. Its role in carbohydrate metabolism is the decarboxylation of Pyruvic acid in the blood and α-ketoacids to acetaldehyde and carbon dioxide. Increased levels of Pyruvic acid in the blood indicate vitamin B_1 deficiency. The chemical name is $C_{12}H_{17}ClN_4OS.HCl$. The molecular formula is Thiazolium, 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl) -4- methyl-, chloride, mono hydrochloride. The molecular weight is 337.27.

Vitamin B_6, also known as Pyridoxine hydrochloride is a water soluble vitamin[5]. Pyridoxine is converted in erythrocytes to Pyridoxal phosphate and to a lesser extent Pyridoxamine phosphate, which act as co-enzymes for various metabolic functions affecting protein, carbohydrate, and lipid utilization. Pyridoxine is involved in conversion of tryptophan to niacin or serotonin, breakdown of glycogen to glucose-1-phosphate, conversion of oxalate to glycine, synthesis of Gamma Aminobutyric acid (GABA). Vitamin B_6 is used to treat Pyridoxine deficiency, including inadequate diet, drug-induced causes (eg, Isoniazid, Hydralazine, oral contraceptives) or inborn errors of metabolism. The Chemical name is 5-Hydroxy-6-methyl-3, 4-pyridinedimethanol hydrochloride. The molecular formula is $C_8H_{11}NO_3.HCl$ and molecular weight is 205.64.

Vitamin B_{12}, also known as cyanocobalamine is used in the body in two forms: Methylcobalamin and 5-deoxyadenosyl cobalamin[6]. The enzyme Methionine synthase needs Methylcobalamin as a cofactor. This enzyme is involved in the conversion of the amino acid homocysteine into Methionine. Methionine in turn is required for DNA Methylation[7]. 5-Deoxyadenosyl cobalamin is a cofactor needed by the enzyme that converts L-methylmalonyl-CoA to succinyl-CoA. This conversion is an important step in the extraction of energy from proteins and fats. Furthermore, succinyl CoA is necessary for the production of hemoglobin, the substances that carries oxygen in red blood cells. Vitamin B_{12} is used to treat vitamin B_{12} deficiency, cyanide poisoning, and hereditary deficiency of transcobalamin II. High vitamin B_{12} level in elderly individuals may protect against brain atrophy or shrinkage associated with Alzheimer's disease and impaired cognitive function[8]. The chemical name is α-(5, 6-dimethyl benzimidazolyl) cobamidcyanide. The molecular formula is $C_{63}H_{89}CoN_{14}O_{14}P$ and molecular weight is 1356.37[9].

The pharmaceutical formulation containing Diclofenac sodium, Vitamin B_1, Vitamin B_6, and Vitamin B_{12} is indicated for women during menopause. Diclofenac sodium acts as analgesic, while Vitamin B_1, Vitamin B_6, and Vitamin B_{12} help to regain the energy lost during menopause. The pharmaceutical formulation (capsules) is marketed by Caplin point laboratories under the brand name Neurotropas".

MATERIALS AND METHOD

Determination of Diclofenac Sodium

The validation was performed using Agilent 1200 series HPLC (Germany) fitted with variable UV-Visible wavelength detector. Diclofenac sodium was obtained from Gujarat Organics. HPLC grade Acetonitrile, Orthophosphoric acid and potassium dihydrogen orthophosphate were obtained from Qualigens.

Chromatographic Condition

Column: Phenomenex ODS C-18 column (150 x 4.6mm; 5µm)

Mobile Phase: Potassium hydrogen phosphate solution of pH 3.0 and Acetonitrile (40:60) Flow rate: 1.0 mL/min

Wavelength : 254 nm

Column temperature: Ambient

Injection volume : 20µL

Run time : 10 minutes

Preparation of Standard Solution

Dissolve accurately 20mg of Diclofenac sodium RS in 100mL volumetric flask, add 50mL of mobile phase to dissolve completely. Dilute to

volume with mobile phase and mix. Filter through 0.2µm finer porosity membrane filter.

Sample Preparation

Take 10 soft gelatin capsules and empty the contents into a small dish and transfer an accurately weighed portion of the mass equivalent to about 20 mg of Diclofenac to a 100 mL volumetric flask. Add 50mL of diluent and mix. Make up the volume to 100mL. Pass a portion of this solution through a filter having a 0.2µm finer porosity, discarding the first 10 mL of the filtrate. Use the clear filtrate as the sample preparation. Calculate the amount of Diclofenac sodium in soft gelatin capsules as follows:

Amount of Diclofenac sodium

= Sample area x Standard weight x100 x Purity (as is) x 100 x Average weight

Standard area x 100 x Sample weight x 100 x Label claim

(Limit: 90.0 to 110.0 %w/w)

Method Validation Parameters

System Suitability

System suitability parameters were evaluated by following ICH guidelines injecting six replicates of 0.2mg/mL concentration of standard Diclofenac Sodium. Resolution factor, theoretical plate and tailing factor were evaluated by following ICH guidelines.

Specificity

Specificity is the degree to which the procedure applies to a single analyte and is checked in each analysis by comparing the blank chromatogram with the chromatogram obtained for the drug spiked with internal standard (placebo) to trace out the interfering peaks. The specificity of the method was investigated by the analysis of the two blank preparation spiked with two different concentration of standard Diclofenac sodium, sample and internal standard (placebo) was also added. The mean, standard deviation and RSD of specificity were calculated.

Linearity & Range

The linearity of the peak area response was determined by making three measurements of an eleven concentration points in the range of 20% to 400% of operating concentration of standard Diclofenac sodium. Standard Area was plotted against the concentration. The linear regression coefficient, correlation coefficient, standard deviation and mean were calculated.

Accuracy

The accuracy was assessed by using a minimum of three different concentration of standard Diclofenac sodium in the range of 100% to 200%. Placebo was spiked into the standard solution of Diclofenac Sodium. The mean, standard deviation and RSD of accuracy were calculated.

Precision

The precision of an analytical method is the closeness of a series of individual measurements of an analyte when the analytical procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision is calculated as coefficient of variation, relative standard deviation. The measure of precision can be subdivided into: repeatability (intra- day precision) and reproducibility.

Reproducibility

Reproducibility of the method assessed by analyzing six times of 0.2mg/mL concentration of standard, Diclofenac sodium solution. The mean, standard deviation and RSD of reproducibility were calculated.

Repeatability

Repeatability of the peak area response was determined by making six measurements at six different concentration points of sample and it was compared with the standard drug Diclofenac sodium. The mean, standard deviation and RSD of repeatability were calculated.

Ruggedness

Ruggedness is defined as the degree of reproducibility of results obtained under a variety of conditions, such as different laboratories, analysts, instruments, environmental conditions, operators and materials. Ruggedness is a measure of reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst. Ruggedness of the method was checked by the analysis of aliquot samples from homogeneous lots by a different chemist on a different instrument on a different day.

Robustness

Robustness was determined by injecting triplicate injection of standards and three sample solutions in single and at different concentration with respect to control condition. Robustness of the method was checked by varying the mobile phase ratio. The sample and standard solutions were injected in each condition. The mean, standard deviation and relative standard deviation of robustness were calculated.

Determination of Vitamin B_1 & B_6

Vitamin B_1 and B_6 was obtained from Gujarat Organics. HPLC grade Acetonitrile, Methanol. Soidum 1-hexane sulphonic acid and glacial acetic acid were obtained from Qualigens.

Chromatographic Condition

Column: Inertsil ODS C-18 column (250 x 4.6mm; 5µm)

Mobile Phase: Mixture of 1000mL of Sodium 1-hexane sulfonic acid solution , 730mL of water, 270mL glacial acetic acid of and 10ml of methanol

Flow rate : 1.5 mL/min

Wavelength : 280 nm

Column temperature: Ambient

Injection volume : 20µL

Run time : 10 minutes

Preparation of Standard Solution

Dissolve accurately 105mg of Vitamin B_1 & 100mg of Vitamin B_6 RS in 100mL volumetric flask. Add 50mL of diluent and sonicate to dissolve completely. Dilute to volume with diluent, and further dilute 5mL to 50mL. Filter through 0.2µm finer porosity membrane filter. A mixture of water, Acetonitrile and Glacial acetic acid (94:5: 1) was used as the diluent.

Sample Preparation

Take 10 soft gelatin capsules and empty the contents into a small dish and transfer 3mL of the sample in a 100mL volumetric flask, dilute to volume with diluent. Filter through 0.45µm Nylon filter. Take 5mL of the filtrate in a 50ml volumetric flask, adjust to volume with diluent. Calculate the amount of Vitamin B_1 and B_6 soft gelatin capsules as follows:

Amount of Vitamin B_1 and B_6

$$= \frac{\text{Sample area x Standard dilution x Purity (as is) x 3 x100}}{\text{Standard area x Sample dilution x 100 x Label claim}}$$

(Limit: 90.0 to 110.0 %w/w)

Method Validation Parameters

System Suitability

System suitability parameters were evaluated by injecting six replicates of 105mcg/mL concentrations of standards Vitamin B_1 & 100mcg/mL Vitamin B_6 respectively. Resolution factor, theoretical plate and tailing factor were calculated.

Specificity

The specificity of the method was investigated by the analysis of the two blank preparation spiked with two different concentrations of standards, Vitamin B_1& Vitamin B_6 sample and internal standard (placebo) was also added. The mean standard deviation and RSD of specificity were calculated.

Linearity & Range

The linearity of the peak area response was determined by making three measurements of an eleven concentration points in the range of 10% to 210% of operating concentrations of standards Vitamin B_1 & Vitamin B_6 respectively. Standard Area was plotted against the concentration. The linear regression coefficient, correlation coefficient, standard deviation and mean were calculated.

Accuracy

The accuracy was assessed by using a minimum of three different concentrations of standards Vitamin B_1 & Vitamin B_6 in the range of 100% to 200%. Placebo was spiked into the standard solutions of Vitamin B_1 & Vitamin B_6. The mean, standard deviation and RSD of accuracy were calculated.

Precision

Reproducibility

Reproducibility of the method assessed by analyzing six times of 105mcg/mL concentrations of standards Vitamin B_1 & 100mcg/mL Vitamin B_6 solution respectively. The mean, standard deviation and RSD of reproducibility were calculated.

Repeatability

Repeatability of the peak area response was determined by making at six measurements at six different concentration of sample and it was compared with the standard drug Vitamin B_1& Vitamin B_6 respectively. The mean, standard deviation and RSD of repeatability were calculated.

Ruggedness

Ruggedness of the method was checked by the analysis of aliquot samples from homogeneous lots by a different chemist on a different instrument on a different day. The sample and standard solutions were injected in each condition, mean, standard deviation and relative standard deviation of robustness were calculated.

Robustness

Robustness was determined by injecting triplicate injection of standards and three sample solutions in single and at different concentration with respect to control condition. Robustness of the method was checked by varying the mobile phase ratio. The sample and standard solutions were injected in each condition, mean, standard deviation and relative standard deviation of robustness were calculated.

Determination of Vitamin B_{12}

Vitamin B_{12} was obtained from Gujarat Organics. HPLC grade Acetonitrile and trifluoroacetic acid were obtained from Qualigens.

Chromatographic Condition

Column: Phenomenex ODS C-18 column (150 x 4.6mm; 5µm)

Mobile Phase: Mixture of 0.25mL trifluroacetic acid, 870mL of water, 130mL of acetonitile

Flow rate : 1.2 mL/min

Wavelength : 361 nm

Column temperature : Ambient

Injection volume : 20µL

Run time : 10 minutes

Preparation of Standard Solution

Dissolve accurately 25mg of Vitamin B_{12} RS in 100mL volumetric flask, add 50mL of mobile phase to dissolve completely, Dilute to volume with mobile phase and further dilute 5mLto 50mL. Filter through 0.2µm finer porosity membrane filter.

Sample Preparation

Take 10 soft gelatin capsules and empty the contents into a small dish and transfer an accurately weighed portion of the mass equivalent to about 0.2 mg of Vitamin B_{12} to a 20mL volumetric flask. Add 10mL of mobile phase and sonicate, then make up the volume to 20mL and mix. Pass a portion of this solution through a filter having a 0.2µm finer porosity,

discarding the first 5 mL of the filtrate. Use the clear filtrate as the sample preparation. Calculate the amount of Vitamin B_{12} in capsules as follows:

Amount of Vitamin B_{12}

= Sample area x Standard weight x 2 x 20 x Purity (as is) x100 x Average weight

Standard area x Sample weight x 100 x Label claim

(Limit: 90.0 to 110.0 %w/w)

Method Validation Parameters

System Suitability

System suitability parameters are evaluated by injecting six replicates of 0.01mg/mL concentration of standards Vitamin B_{12}. Resolution factor, theoretical plate and tailing factor were calculated.

Specificity

The specificity of the method was investigated by the analysis of the two blank preparation spiked with two different concentrations of standard Vitamin B_{12} and internal standard (placebo) was also added. The mean standard deviation and RSD of specificity were calculated.

Linearity & Range

The linearity of the peak area response was determined by making three measurements of a eleven concentration points in the range of 10% to 200 % of operating concentrations of standards Vitamin B_{12}. Standard Area was plotted against the concentration. The linear regression coefficient, correlation coefficient, standard deviation and mean were calculated.

Accuracy

The accuracy was assessed by using a minimum of three different concentrations of standard Vitamin B_{12} in the range of 100 to 200%. Placebo was spiked into the standard solutions of Vitamin B_{12}. The mean, standard deviation and %RSD of accuracy were calculated.

Precision

Reproducibility

Reproducibility of the method assessed by analyzing six times of 0.01mg/mL concentrations of standard Vitamin B_{12} solution. The mean, standard deviation and %RSD of reproducibility were calculated.

Repeatability

Repeatability of the peak area response was determined by making at six measurements at six different concentration of sample and it was compared with the standard drug Vitamin B_{12}. The mean, standard deviation and RSD, of repeatability were calculated.

Ruggedness

Ruggedness of the method was checked by the analysis of aliquot samples from homogeneous lots by a different chemist on a different instrument on a different day. The sample and standard solutions were injected in each condition, mean, standard deviation and relative standard deviation, of robustness were calculated.

Robustness

Robustness was determined by injecting triplicate injection of standards and three sample solutions in single and at different concentration with respect to control condition. Robustness of the method was checked by varying the mobile phase ratio. The sample and standard solutions were injected in each condition, mean, standard deviation and relative standard deviation of robustness were calculated.

RESULTS AND DISCUSSION

Diclofenac Sodium

System Suitability

The system suitability parameters such as retention time, number of theoretical plates and peak area response were also be calculated for the standard drug solution and mentioned in Table 1. It was observed that all the values are within the limits.

Table1: System suitability parameters for Diclofenac sodium

S.No.	System suitability parameters	Results
1.	Retention time	5.428 minutes
2.	Peak area response	70431454
3.	Theoretical plates	11292

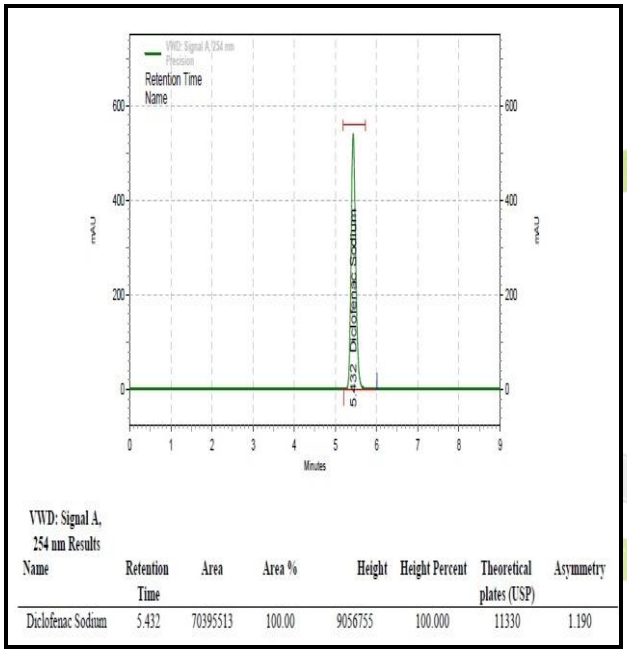

Figure 1: Typical chromatogram of Diclofenac sodium

Specificity

The specificity of the HPLC method was that complete separation of Diclofenac sodium was noticed in presence of other inactive excipients used in capsule formulation. In addition, there was no any interference at the retention time in the chromatogram of placebo solution. In peak purity analysis with PDA, purity angle was always less than purity threshold for the analyte. This shows that the peaks of analyte were pure and excipients in the formulation does not interfere the analyte.

Table 2: Specificity for Diclofenac sodium

S.No.	Name	No. of Injections	Area
1.	Blank	1	Nil
2.	Placebo	1	Nil
3.	Standard	1	70431454
4.	Sample	1	71348903

Linearity and Range

The Linearity of this method was determined at eleven levels from 20% – 400% of operating concentrations for Diclofenac sodium. The plot of peak area of each sample against respective concentration of Diclofenac sodium was found to be linear (Figure 2) in the range of 20% – 400% of operating concentrations. Beer's law was found to be obeyed over this concentration range. The linearity was evaluated by linear regression analysis using least square method. The regression equations were found to be Y=37555x - 52347 for Diclofenac sodium and correlation coefficient of the standard curves were found to be 0.99999 for Diclofenac sodium. It observed that correlation coefficient and regression analysis are within the limits.

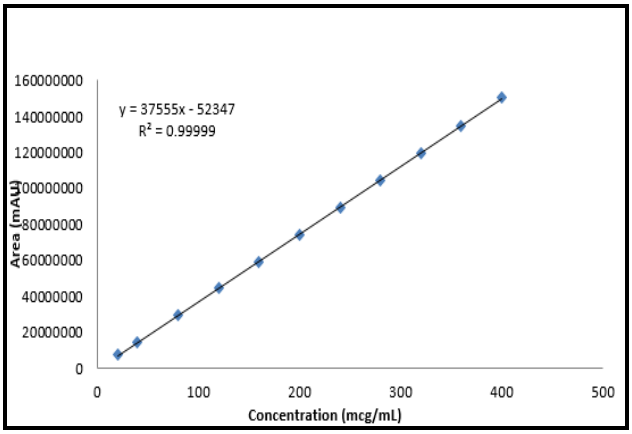

Figure 2: Linearity of response for Diclofenac sodium

Accuracy

Accuracy of the method was found out by recovery study by standard addition method. The known amounts of standard Diclofenac sodium was added to pre-analyzed samples at a level from 100% up to 200% and then subjected to the proposed HPLC method individually. The results of recovery studies were shown in Table 3. It was observed that the mean percentage recoveries found for Diclofenac sodium demonstrated that the method was highly accurate.

Table 3: Accuracy of response for Diclofenac sodium

Solution	Quantity added (Known) µg/mL	Area	% Recovery
Solu.-1	140	54677470	100.00
Solu.-2	168	65903546	100.44
Solu.-3	196	77214247	100.87
Solu.-4	224	88631052	101.31
Solu.-5	252	100258439	101.87
Solu.-6	280	111392035	101.86

Precision

Reproducibility

Reproducibility data for Diclofenac sodium is shown in Table 4. The %RSD was 0.05%. This indicated that method was highly precise.

Table 4: Precision - Reproducibility for Diclofenac sodium

S.No	Sample	Area
1	Sample-1	70395513
2	Sample-2	70400159
3	Sample-3	70476190
4	Sample-4	70436657
5	Sample-5	70407211
6	Sample-6	70472993
Mean		**70431454**
% RSD		**0.05**

Repeatability

Repeatability data for Diclofenac sodium is shown in Table 5. The %RSD was 0.07.This indicated that method was highly precise.

Table 5: Precision - Repeatability for Diclofenac sodium

S.No	Sample	Area
1	Sample-1	71402736
2	Sample-2	71401164
3	Sample-3	71346783
4	Sample-4	71345795
5	Sample-5	71289915
6	Sample-6	71307025
	Mean	**71348903**
	% RSD	**0.07**

Ruggedness

The ruggedness data of Diclofenac sodium is shown in Table 6. It was observed that there were no marked changes in the chromatograms, which demonstrated that the proposed method was robust.

Table 6: Ruggedness data for Diclofenac sodium -Change of analyst

S.No	Sample	Area
1.	Sample-1	70573632
2.	Sample-2	70571299
3.	Sample-3	70571888

4.	Sample-4	70571757
5.	Sample-5	70599546
6.	Sample-6	70567925
Mean		**70576008**
% RSD		**0.02**

Robustness

The robustness of the method was demonstrated by changing the ratio of solvents in the mobile phase. Two sample preparations were analyzed as per methodology by changing the ratio of solvents in the mobile phase by means of ± 2.0%. The data is presented in Table 7. It was observed that there were no marked changes in the area, which demonstrated that the proposed method was robust.

Table 7: Robustness - Change in the ratio of solvents in the mobile phase (- 2%) for Diclofenac sodium [Buffer: Acetonitrile (38:62)]

S.No.	Sample Name	Peak Area
1.	Sample -1	70563634
2.	Sample -2	70574513
Mean		**70569074**
% RSD		**0.01**

Vitamin B_1 & B_6

System Suitability

The system suitability parameters such as retention time, number of theoretical plates and peak area response were also calculated for the standard drug solution and mentioned in Table 8. It was observed that all the values are within the limits.

Specificity

The specificity of the HPLC method was that complete separation of Vitamins B_1 and B_6 was noticed in presence of other inactive excipients used in capsule formulation. In addition, there was no any interference at the retention time in the chromatogram of placebo solution. In peak purity analysis with PDA, purity angle was always less than purity threshold for the analyte. This shows that the peaks of analyte were pure and excipients in the formulation does not interfere the analyte.

Table 8: System suitability for Vitamin B_1 & B_6

System suitability parameters	Vitamin B_1	Vitamin B_6
Retention time	3.701min	7.72 min
Peak area response	286657752	145560207
Theoretical plate	8967	7521

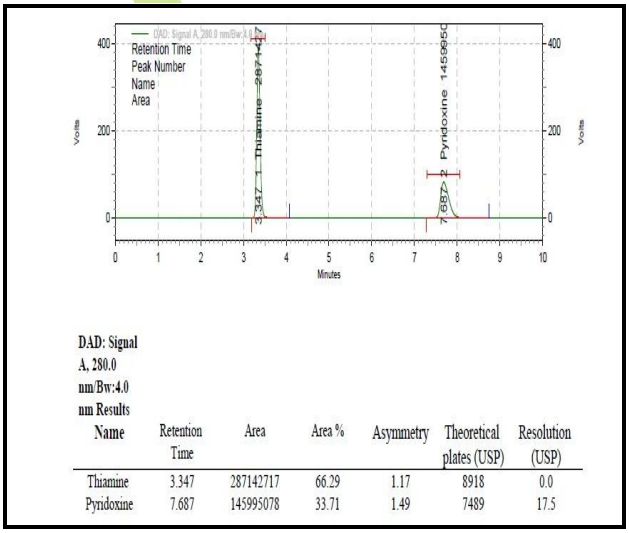

Figure 3: Typical chromatogram of Vitamin B_1 and B_6

Table 8A: Specificity for Vitamin B_1 and B_6

Name	No. of Injections	Area
Blank	1	Nil
Placebo	1	Nil

Standard (Vitamin B_1)	1	70431454
Sample (Vitamin B_1)	1	71348903
Standard (Vitamin B_6)	1	145560207
Sample (Vitamin B_6)	1	186541390

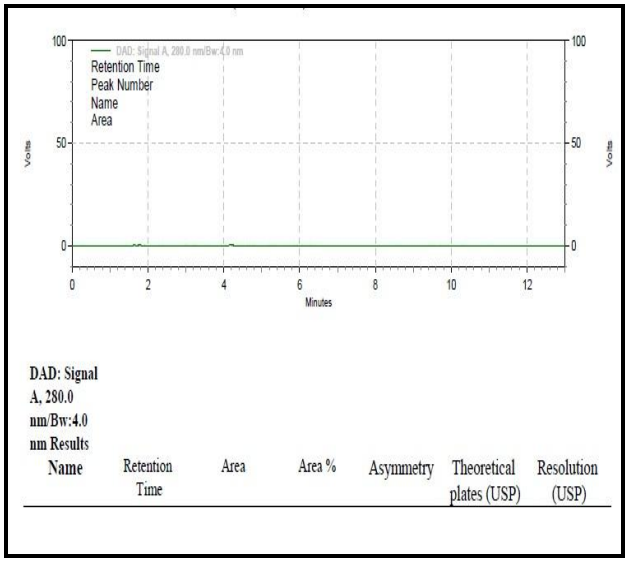

Figure 4: Chromatogram for the study of specificity of blank

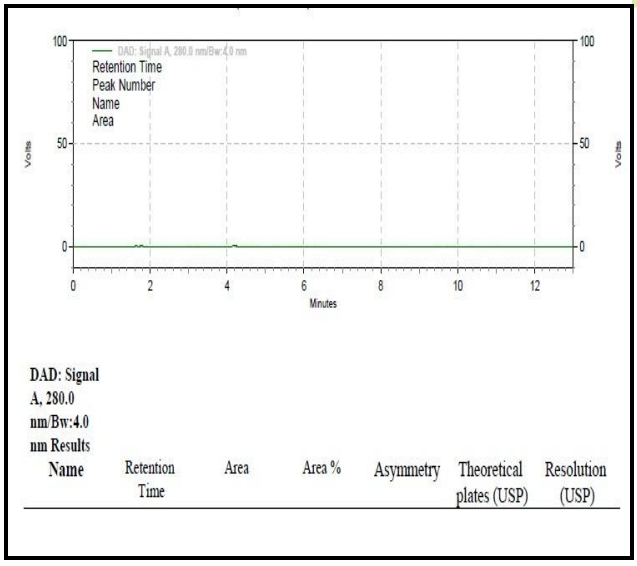

Figure5: Chromatogram for the study of specificity of placebo

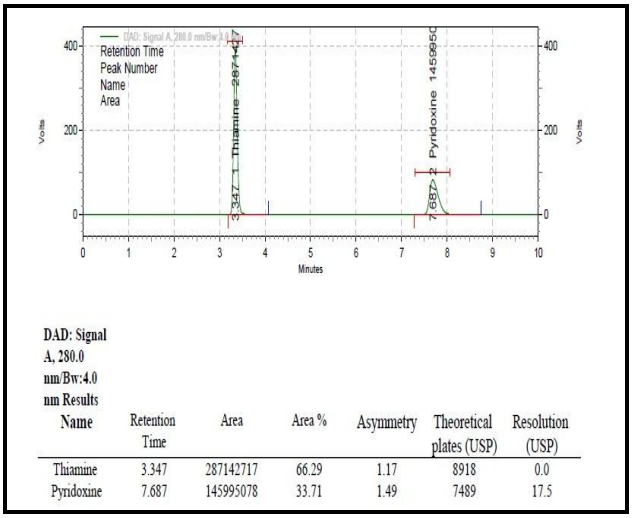

Figure 6: Chromatogram for the study of specificity of Standard

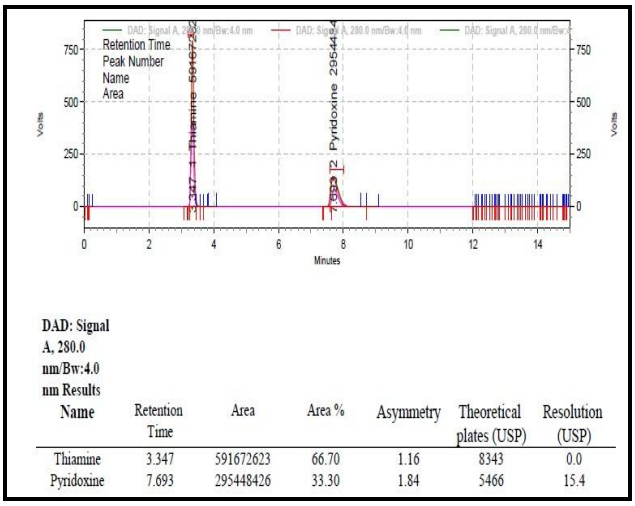

Figure 7: Chromatogram for the study of specificity of sample

Linearity and Range

The plot of peak area of each sample against respective concentrations of Vitamin B_1 & Vitamin B_6 were found to be linear (Figures 8 and 9) in the range of 10%– 200% of operating concentrations. Beer's law was found to be obeyed over this concentration range. The linearity was evaluated by linear regression analysis using least square method. The regression equations were found to be Y=2,865,536.3707x + 1,738,653.5726 and Y=14,37,350.5727x + 16,03,142.8436 for Vitamin B_1 & Vitamin B_6 and correlation coefficient of the standard curves were found to be 0.99997 and 0.9998 for Vitamin B_1 &

Vitamin B_6 respectively. It observed that correlation coefficient and regression analysis are within the limits.

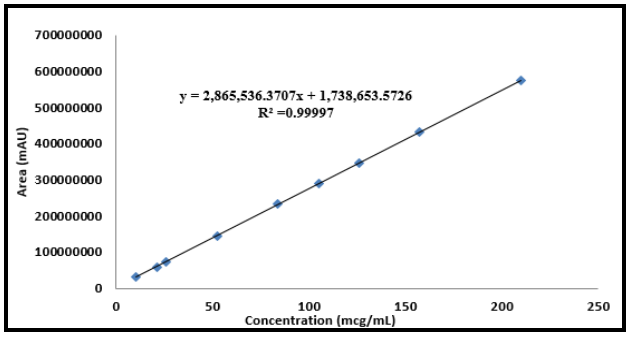

Figure 8: Linearity of response for Vitamin B_1

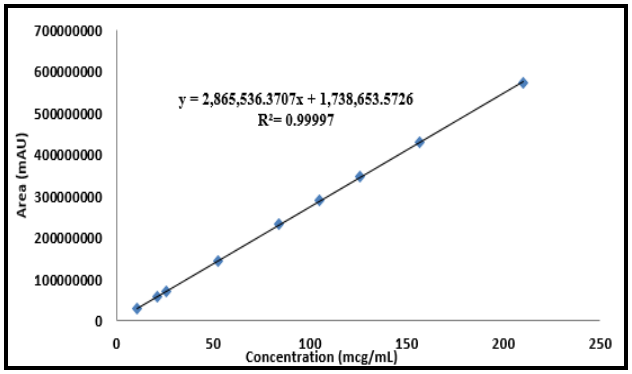

Figure 9: Linearity of response for Vitamin B_6

Accuracy

Accuracy of the method was found out by recovery study by standard addition method. The known amounts of standards, Vitamin B_1 and B_6 were added to pre-analyzed samples at a level from 100% up to 200% and then subjected to the proposed HPLC method individually. The results of recovery studies were shown in Table 9. It was observed that the mean percentage recoveries were found to be for Vitamin B_1 and B_6 which demonstrated that the method was highly accurate.

Table 9: Accuracy for Vitamin B_1

Solution	**Quantity added (Known) µg/mL**	**Area**	**% Recovery**
Solu-1	100	203663196	100.00
Solu -2	115	232939824	99.46
Solu -3	130	262035522	98.97
Solu -4	140	290875524	102.02
Solu-5	160	329221160	101.03
Solu-6	170	348327318	100.61
Solu -7	185	377110935	100.09
Solu-8	200	407033426	99.93

Table 10: Accuracy for Vitamin B6

Solution	**Quantity added (Known) µg/mL**	**Area**	**% Recovery**
Solu-1	100	101774521	100
Solu -2	115	116283316	99.35
Solu -3	130	130735288	98.81
Solu -4	140	145020887	101.78
Solu -5	160	159445650	98.92
Solu -6	170	173727762	100.41
Solu -7	185	188080328	99.89
Solu-8	200	202508127	99.49

Precision

Reproducibility

Reproducibility data for vitamin B_1 and B_6 is shown in Table 11. The %RSD was 0.34% and 0.17 % for vitamin B_1 and B_6 respectively. This indicated that method was highly precise.

Repeatability

Repeatability is the precision of a method under the same operating conditions over a short period of time. One aspect of this is instrumental precision. A second aspect is sometimes termed intra-assay precision and involves multiple measurements of the same

sample by the same analyst under the same conditions. Repeatability data for vitamin B_1 and B_6 were shown in Table 12. This indicated that method was highly precise.

Table 11: Precision - Reproducibility for Vitamin B_1 and B_6

Replicates	Area of Vitamin B_1	Area of Vitamin B_6
Sample-1	287142717	145995078
Sample-2	287490457	145652695
Sample-3	285551325	145345304
Sample-4	285987577	145436109
Sample-5	285907247	145348939
Sample-6	287867187	145583122
Mean	**286657752**	**145560208**
% RSD	**0.34**	**0.17**

Table 12: Precision - Reproducibility for Vitamin B_1 and B_6

Replicates	Area	Area
Sample-1	71402736	71402736
Sample-2	71401164	71401164
Sample-3	71346783	71346783
Sample-4	71345795	71345795
Sample-5	71289915	71289915
Sample-6	71307025	71307025
Mean	**370892469**	**186541390**
% RSD	**0.05**	**0.07**

Ruggedness

Six sample preparations were analyzed as per the methodology by a different analyst on a different instrument on a different day. The ruggedness data of vitamin B_1 and B_6 were shown in Table 13. It was observed that there were no marked changes in the chromatograms, which demonstrated that the proposed method was robust.

Table 13: Ruggedness data for Vitamin B_1 and B_6 -Change of analyst

Replicates	Area of Vitamin B_1	Area of Vitamin B_6
Sample-1	296289559	148721741
Sample-2	296545840	149010606
Sample-3	296324710	148921465
Sample-4	296445701	148786843
Sample-5	296461388	148782386
Sample-6	296465287	148817069
Mean	**296422081**	**148840018**
% RSD	**0.03**	**0.07**

Robustness

The robustness of the method was demonstrated by changing the ratio of solvents in the mobile phase. Two sample preparation were analyzed as per methodology by changing the ratio of solvents in the mobile phase by means of ±2.0 % . The data is presented in Table 14. It was observed that there were no marked changes in the area, which demonstrated that the proposed method was robust.

Table 14: Robustness - Change in the ratio of solvents in the mobile phase (- 2%) for Vitamin B_1 and B_6 [Water: Acetonitrile (71:29)]

Sample	Area for Vitamin B_1	Area for Vitamin B_6
Sample -1	296465287	148721741
Sample -2	296324770	149016061
Mean	**296395029**	**148868901**
% RSD	**0.34**	**0.14**

Vitamin B_{12}

System Suitability

The system suitability parameters such as retention time, number of theoretical plate and peak area response were also be calculated for the standard drug solution and mentioned in Table 15. It was observed that all the values are within the limits.

Table 15: System suitability parameters for Vitamin B_{12}

S.No.	System suitability parameters	Results
1.	Retention time	6.76 min
2.	Peak area response	3444712
3.	Theoretical plate	794

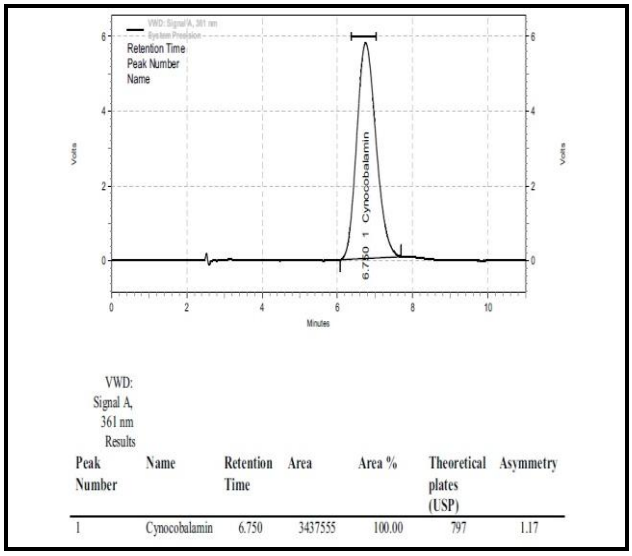

Figure 10: Typical chromatogram of Vitamin B_{12}

Specificity

The specificity of the HPLC method was that complete separation of Vitamin B_{12} was noticed in presence of other inactive excipients used in capsule formulation. In addition, there was no any interference at the retention time in the chromatogram of placebo solution. In peak purity analysis with PDA, purity angle was always less than purity threshold for the analyte. This shows that the peaks of analyte were pure and excipients in the formulation does not interfere the analyte.

Table 16: Specificity for Vitamin B_{12}

S.No.	Name	No. of Injections	Area
1.	Blank	1	Nil
2.	Placebo	1	Nil
3.	Standard	1	70431454
4.	Sample	1	71348903

Linearity and Range

The Linearity of this method was determined at eleven levels from 10%–200% of operating concentrations for Vitamin B_{12}. The plot of peak area of each sample against respective concentration of Vitamin B12 were found to be linear. Beer's law was found to be obeyed over this concentration range. The linearity was evaluated by linear regression analysis using least square method. The regression equations were found to be Y=3,41,841.1712x + 16,394.6474 for Vitamin B_{12} and correlation coefficient of the standard curves were found to be 0.9999 for Vitamin B_{12}. It observed that correlation coefficient and regression analysis are within the limits.

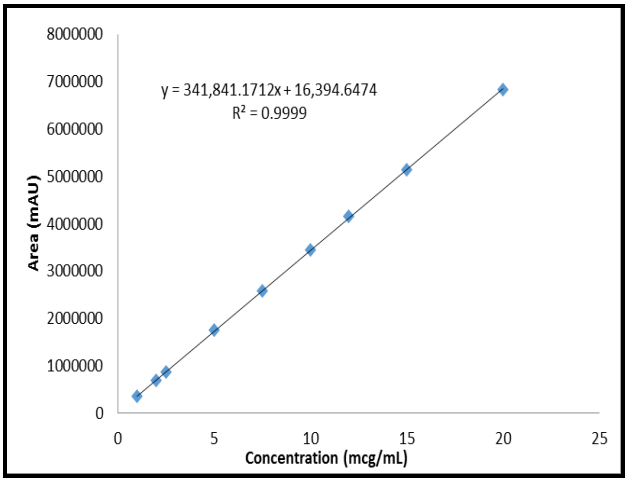

Figure 11: Linearity of response for Vitamin B_{12}

Accuracy

The known amounts of standard Vitamin B_{12} were added to pre-analyzed samples at a level from 100% up to 200% and then subjected to the proposed HPLC method. The mean percentage recoveries were found to be within limits for Vitamin B_{12} which demonstrated that the method was highly accurate. The results are shown in Table 17.

Table 17: Accuracy of response for Vitamin B_{12}

Solution	Conc. added (µg/mL)	Area	% Recovery
Solu-1	7	5738752	100.00
Solu-2	8	6505746	98.86
Solu-3	9	7389618	99.18
Solu-4	10	8513664	99.03
Solu-5	11	9281849	101.27
Solu-6	12	10305111	99.93
Solu-7	13	10978493	100.94
Solu-8	14	11670252	101.93

Precision

Reproducibility

Reproducibility data for Vitamin B_{12} is shown in Table 18. The %RSD was 0.62%. This indicated that method was highly precise.

Table 18: Precision - Reproducibility for Vitamin B_{12}

S.No	Sample	Area
1	Sample-1	3437555
2	Sample-2	3467728
3	Sample-3	3469258
4	Sample-4	3445447
5	Sample-5	3413547
6	Sample-6	3434737
Mean		**70431454**
% RSD		**0.62**

Repeatability

Intra-assay precision was performed by multiple measurements of the same sample by the same analyst under the same conditions. Repeatability data for Vitamin B_{12} were shown in Table 19. This indicated that method was highly precise.

Table 19: Precision - Repeatability for Vitamin B_{12}

S.No	Sample	Area
1	Sample-1	4666015
2	Sample-2	4616369
3	Sample-3	4636558
4	Sample-4	4673274
5	Sample-5	4630055
6	Sample-6	4639325
Mean		**4643599**
% RSD		**0.47**

Ruggedness

Six sample preparations were analyzed as per the methodology by a different analyst on a different instrument on a different day. The ruggedness data of Vitamin B_{12} were shown in Table 20. It was observed that there were no marked changes in the chromatograms, which

demonstrated that the proposed method was rugged.

Table 20: Ruggedness data for Vitamin B_{12}- Change of analyst

S.No	Sample name	Area
1.	Sample-1	3550107
2.	Sample-2	3596384
3.	Sample-3	3560557
4.	Sample-4	3556322
5.	Sample-5	3534743
6.	Sample-6	3566471
Mean		**3560764**
% RSD		**0.08**

Robustness

The robustness of the method was demonstrated by changing the ratio of solvents in the mobile phase. Two sample preparation were analyzed as per methodology by changing the ratio of solvents in the mobile phase by means of ±2.0 % . The data is presented in Table 21. It was observed that there were no marked changes in the area, which demonstrated that the proposed method was robust.

Table 21: Robustness - Change in the ratio of solvents in the mobile phase (- 2% for Vitamin B_{12} [Buffer: Acetonitrile (85:15)]

S.No.	Sample Name	Peak Area
1.	Sample -1	6095632
2.	Sample -2	6052871
Mean		**6052871**
% RSD		**0.99**

CONCLUSION

The proposed study describes new and simple RP-HPLC method for the estimation of Diclofenac sodium, Vitamin B_1, Vitamin B_6 and Vitamin B_{12} in soft gelatin capsule dosage form. The method was validated as per ICH guidelines and found to be simple, sensitive, accurate and precise. Therefore the proposed method can be successfully used for the routine lab analysis of estimation of Diclofenac sodium, Vitamin B_1, Vitamin B_6, and Vitamin B_{12} in soft gelatin capsule dosage form. Furthermore the test specificity proved that this method can be applied for estimation of all drug contents without any excipients interference. The obtained chromatograms showed good resolutions between the peaks which helps to quantify the drugs in well-defined manner.

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