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Development and validation of a RP-HPLC method for simultaneous estimation of pyridoxine hydrochloride (Vitamin B6) and its degraded products formed under effect of different solvents

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ABSTRACT

The aim of the present study was to develop a relatively simple, sensitive, validated, and reliable HPLC method for the determination of pyridoxine hydrochloride substance and drug product. Very simple, rapid, sensitive, accurate, and less expensive HPLC method was develop for the simultaneous estimation of pyridoxine hydrochloride (vitamin B6). Pyridoxine hydrochloride has absorbance at wavelength 254 nm in a mixture of potassium dihydrogen phosphate (pH 3±0.2) and methanol with a ratio 70:30 respectively, mobile phase was delivered at the flow rate of 1.0 ml/ min. Substance was resolved on a C18 column (thermo 250*4.6 mm id, 5 µm).

The linearity was obtained over the concentration ranges 10-50µg/ml with linear regression (r^2) = 0.9996. Separation completed within 3.5 min ± 0.02, no interference for any excipients observed. Accuracy (recovery) calculated as percentage found to be between 98.8 – 100.86 %. Method found to be reproducible with relative standard deviation (RSD) for intra and interday precision < 2 over the concentration range. The results obtained from the analysis of pyridoxine by developed validated method were successfully to the determination of pyridoxine substance, drug product and it can be useful for their combination.

KEY WORDS: Pyridoxine hydrochloride, HPLC, development and validation, Analysis, stability.

1.INTRODUCTION

Vitamin B6 consists of three interconvertible pyrimidine vitamers: Pyridoxine, which is occurring naturally in plant products, pyridoxal, and pyridoxamine, which are occur naturally in animal tissues), and their phosphate esters (Sampson D.A., O'Connor D.K, 1989; Nollet L.M, 2004; Hermann W, Obeid R, 2011). The form of Vitamin B6 that most often used in supplements is Pyridoxine hydrochloride, and it is the least expensive form to produce commercially (Haas E.M, 2006). Pyridoxine hydrochloride occurs in the human body by the enzyme pyridoxal kinase as pyridoxal phosphate, which acts as a coenzyme in more than 100 enzymatic reactions in the metabolism of amino acids, carbohydrates, and fats (Toney M.D, 2005). It plays role in reducing the complications caused by diabetes, age and neurodegenerative diseases (Insel p, 2002). It also serving as regulators of membrane ion transporters (Lambrecht G, 2002) and it is recommended in treatment of intractable seizures (Ohtahara, 2011). Chemically, Pyridoxine hydrochloride name is 2-methyl-3-hydroxy-4, 5-bis (hydroxymethyl) pyridine hydrochloride (Figure 1.) and its molecular mass is 205.64. It is a white powder that is freely soluble in water (1g/5ml) and stable in air, heat and acid solutions, while it is unstable in alkaline medium and light (Marz R.B, 1999).

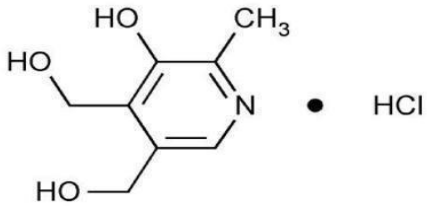

Fig.1.Structural formula of Pyridoxine hydrochloride

In this work was to develop a relatively simple, sensitive, validated, and reliable HPLC method is establish to assay of Pyridoxine hydrochloride substance and drug products.

2. MATERIALS AND METHODS

2.1 Materials (Chemicals and Reagents): Pyridoxine hydrochloride was gift from DSM Company, all chemicals and regents used were of a HPLC grade. Potassium dihydrogen phosphate was obtained from Applichem, Germany. Methanol was obtained from fisher scientific UK Limited, UK. Water (HPLC gradient grade) supplied from Panreac, E.U. Orthophasophoric acid 85 % was obtained from BDH, England.

2.2 Instrument and Equipment:

a. HPLC instruments a water Breeze 2 system, consisting of binary pump series 1525, UV/VIS detector 2489, and auto sampler series 2707.

b. Sensitive balance, A&D Company limited, Japan.

c. 827 pH lab. metrohm ion analysis, Herisau/ Switzerland.

2.3 Experimental:

2.3.1 Preparation of 0.015M potassium dihydrogen phosphate pH 3: Potassium dihydrogen phosphate was accurately weighed 2.055 gram and transferred to a 1000ml volumetric flask, and dilute up to mark by distilled water and sonicated. The solution was adjusted to pH 3 with diluted Orthophospharic acid.

2.3.2 Preparation of Mobile phase: The mobile phase was prepared by mixing a solution of 0.015 M Potassium dihydrogen phosphate (pH 3 ± 0.2) and methanol with a ratio 70:30 respectively and degased.

2.3.3. Chromatographic conditions used for the analysis of the drug: Several factors were selected through a number of trials conducted as optimum conditions for the analysis of pyridoxine hydrochloride. Column C18 (Thermo – hypersil GOLD), 250 mm × 4.6 mm i.d., particle size 5 µm was used for separation. Mobile phase used for separation was mixture of 0.015M Potassium dihydrogen phosphate pH 3 and acetonitrile with a ratio of 7:3 respectively, column temperature was ambient (25°C). The flow rate was 1.0 mL/min, eluents were detected by UV detector at 254 nm, and the injection volume was 30 µL.

2.3.4 Preparation of standard stock solution: Stock standard solution having concentration 0.5 mg/ml was prepared by dissolving pure drug of pyridoxine hydrochloride in mobile phase.

2.3.5. Method validation

2.3.5.1. Specificity: Specificity of the method was determined by comparison between standard drug and sample. Concentrations of 100 µg/mL of standard and working test solutions were injected to the HPLC system for six replicates and were analysed. Percentage of RSD was calculated from their peak height.

2.3.5.2 System Suitability Testing: System suitability testing is used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed, was carried out for concentrations of 100 µg/mL of standard (% RSD for retention time and for peak height of six replicates) are determined (Table 1).

2.3.5.3 Dilution for linearity studies (standard calibration curve): The calibration curve was prepared by using the standard stock solution with dilution to get concentration of 10, 20, 30, 40, and 50µg/ml (Figure 2).

2.3.5.4 Determination Limit of Detection (LOD) and Limit of Quantitation (LOQ): LOD and LOQ of Pyridoxine hydrochloride was determined by calibration curve method. Solutions of pyridoxine hydrochloride were prepared in the range of 10–50 µg/mL and injected in triplicate.

Limit of detection can be calculated by using the following formula [8, 9]:

$$LOD = \frac{3.3\,\sigma}{S}$$

Limit of quantitation can be calculated based on standard deviation of the response and the slope.

$$LOQ = \frac{10\,\sigma}{S}$$

Where σ = Standard deviation of the response.

S = Slope of the calibration curve.

2.3.5.5 Preparation Solution for Accuracy and Recovery Studies: To study the accuracy of the developed method and to check the interference from excipients, recovery studies carried out by addition of standard drug solution to sample at three different level (80 – 120 %) of the test concentration (3*3). Recovery study were carried out by adding accurately weighing amounts of pyridoxine hydrochloride to the excipients mixture and calculating the percentage recovery in each case (Table 3).

2.3.5.6 Preparation solution for precision studies: Precision of the method checked by six replicate reading of a concentration of 100 µg/ml of pyridoxine hydrochloride as repeatability precision and 3 replicate for readings by three different analysts for a concentration of 100 µg/ml as intermediate precision (Table 4,5,6,and 7).

2.3.5.7 Robustness Studies: Robustness of the method was determined by small changes in flow rate, mobile phase ratio, pH and wave length of detection. Flow rate was changed to 1 ± 0.2 ml/minute, mobile phase ratio was changed to ±5% for both components, pH (3 ± 0.2) Wave length of detection was changed to 254 ± 2 nm.

2.3.5.8 Preparation test sample for the assay of marketed pyridoxine: For determination of the content pyridoxine hydrochloride tablet 40 mg and pyridoxine hydrochloride 50 mg combined with meclizine hydrochloride 25 mg tablet, weighed out accurately a weight of tablet equivalents to 40 mg of pyridoxine hydrochloride and 50 mg of pyridoxine hydrochloride combined, transferred to a 100 ml volumetric flask. The volume was made up to 90 ml with mobile phase and sonicated for 30 minute, completed up to mark with the same mobile phase, further dilution was made from this solution to get concentration of pyridoxine hydrochloride 10µg / ml (Table 8**).**

2.3.5.9 Stability studies

2.3.5.9.1 Sample preparation of pyridoxine hydrochloride submitted into neutral media (water): 100 µg/ml water of pyridoxine hydrochloride was prepared; 2 ml was diluted to a 100 ml with HPLC water. 30 µl of the sample was injected into the chromatographic column.

2.3.5.9.2 Sample preparation of pyridoxine hydrochloride submitted into acidic media (0.1M HCl) at room temperature: 100 µg/ml 0.1M HCl of pyridoxine hydrochloride was prepared, 2 ml was diluted to a 100 ml with HPLC water. 30 µl of the sample was injected into the chromatographic column.

2.3.5.9.3 Sample preparation of pyridoxine hydrochloride submitted into alkaline media (0.1M NaOH) at room temperature: 100 µg/ml 0.1M NaOH of pyridoxine hydrochloride was prepared, 2 ml was diluted to a 100 ml with HPLC water. 30 µl of the sample was injected into the chromatographic column.

3. RESULTS AND DISCUSSION

Different types of mobile phases were used through development HPLC method for estimation of Pyridoxine hydrochloride. The use of 0.015 M potassium dihydrogen phosphate (pH=3±0.2) and methanol with a ratio of 70:30 respectively ,detection wavelength 254 nm and flaw rate 1.0 ml/min for 30 µl of sample injected were preferred as its used for good resolution and peak shape which completed within 3.5 minutes for pyridoxine hydrochloride (Figure.2).

Specificity was found to be RSD < 2(Table 1) which complies with ICH guidelines.

With selected method parameters, system suitability testing provided good resolution and reproducibility and was adequate for analysis to be performed for the resolution of pyridoxine hydrochloride (Table 2).

Linearity was observed over concentration of 10 – 50 µg / ml (Figure 3) has shown good linearity (r^2 =0.9996), which is within the limits of ICH guidelines and the method's linearity proved to be reproducible. LOD and LOQ of pyridoxine hydrochloride was determined by calibration curve method. Solutions of pyridoxine hydrochloride were prepared in the range of 10–50 µg/mL and injected in three replicate (Table 3). The low values obtained of LOD and LOQ indicates that method can be used for detection and quantification of pyridoxine hydrochloride over a very wide range of concentrations.

For accuracy studies which calculated as recovery studies (Table 4), determined by using three different concentrations (80%, 100%, and 120%) of pyridoxine hydrochloride. The RSD % values were found 0.44 % and recovery was 100.02%. Precision (repeatability, intermediate) was carried out by using a 100µg/ml as concentration with six replicates as intermediate precision and three replicates spiked for three different analysts as intermediate precision. For all levels RSD % is less than 2.0 % (Table 5**)** value indicated that proposed method were accurate and precise. Robustness for the developed RP-HPLC method for the estimation of pyridoxine hydrochloride found to be robust as the RSD% found to be < 2 (Table 6), which is the limit as per ICH guidelines and hence the method proved to be robust.Good resolution obtained when analyzed the combine of pyridoxine HCl and meclozine HCl by developed validated method (Figure 4). Marketed formulation of pyridoxine hydrochloride and their combined dosage were determined. Relative concentrations of two products were calculated (Table 7).

Stability studies of pyridoxine hydrochloride carried out under effect of acidic solvent (0.1M HCl for 8 hour), it found that a drug completed degraded and detectable as one degraded peak. The drug submitted also to alkaline solvent (0.1M NaOH for 8 hour) ,it found all drug degraded and shows 2 detectable peak(s) ,one of them related to that detectable in acidic solvent. The drug observed more stable in neutral solvent. The results of effects different solvent are shown in (Table 8) and (Figures 5,6,7); the results obtained revealed that pyridoxine hydrochloride was instable in acidic and alkaline media.

Table.1.Specificity data

No. of injection	Mean Peak height (standard)	Mean Peak height (sample)	% RSD
6	690100.504	683325.335	0.49

Table.2.System suitability parameters

Parameters	Value (Mean ± % RSD)*
Peak height	687260±0.96
Retention time (R.T minutes)	3.5 ±0.1
Theoretical plate (N)	29054±0.14
Tailing factor	0.81± 0.01

***Mean and %RSD for six replicate.**

Table.3.Results of LOD and LOQ of pyridoxine hydrochloride

Range	Linear equation	Linear regression R2	LOD	LOQ
10- 50 µg/ml	Y= 1726.8 X	0.9996	0.38 µg/ml	1.16 µg/ml

Table.4.Test results for accuracy studies (Recovery) of pyridoxine hydrochloride

Run	% Amount addition	%Amount found	% Recovery	RSD
1	80	79.55	100.55	0.46
2	80	80.30	99.63	
3	80	79.93	100.09	
1	100	101.21	98.80	0.63
2	100	101.99	99.01	
3	100	100.03	99.97	
1	120	119.23	100.63	0.13
2	120	119.25	100.62	
3	120	118.93	100.86	
Accuracy = Mean over all recovery			100.02%	0.41

Table.5.Results for repeatability and intermediate precision

Precision	RSD
Repeatability	0.46%
Intermediate precision method (carried out byX-analyst)	1.10%
Intermediate precision method (carried out by Y-analyst)	0.93%
Intermediate precision method (carried out by Z-analyst)	0.44%
Overall RSD	0.73 %

Table.6.Results of robustness

Parameters	% RSD
Flow rate (±0.2 ml/ min)	
0.8 ml/min	0.21
1.0 ml/min	0.06
1.2 ml/min	0.34
Wavelength (± 2 nm)	
252 nm	0.43
254 nm	0.12
256 nm	0.55

Table.7.Results for the analysis marketed products of pyridoxine

Marketed tablet	Label claim (mg/ tablet	Amount found (mg / tablet)	% Claim# ± SD
I	40	39.98	99.9 ± 0.14
ii	50	50.03	100.06 ± 0.45

I Mean pyridoxine hydrochloride tablet 40 mg(Julphar), **ii** = pyridoxine hydrochloride 50 mg combined with meclizine 25 mg tablet(Navidoxine,GlaxoSmithkline) , # = of five determination for each product.

Table.8.Test results for the effect of different media on the stability of pyridoxine hydrochloride

Peak name	Drug submitted into different solvent (R.T minute)		
	Water	0.1M HCl	0.1M NaOH
Pyridoxine hydrochloride	3.49	-	-
Decomposed 1	0	6.49	6.48
Decomposed 2	0	0	9.09

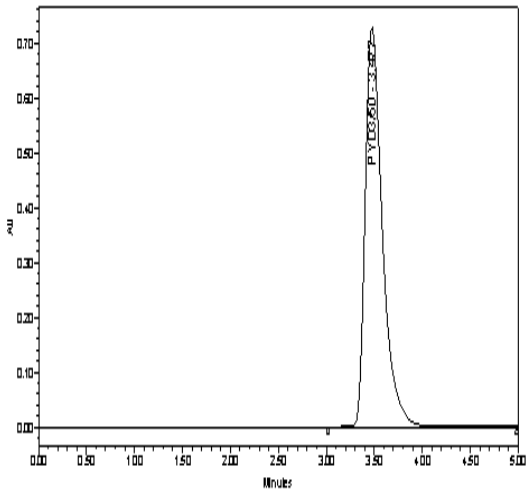

Figure.2.Optimized HPLC chromatogram test for the analysis of pyridoxine hydrochloride standard by developed method

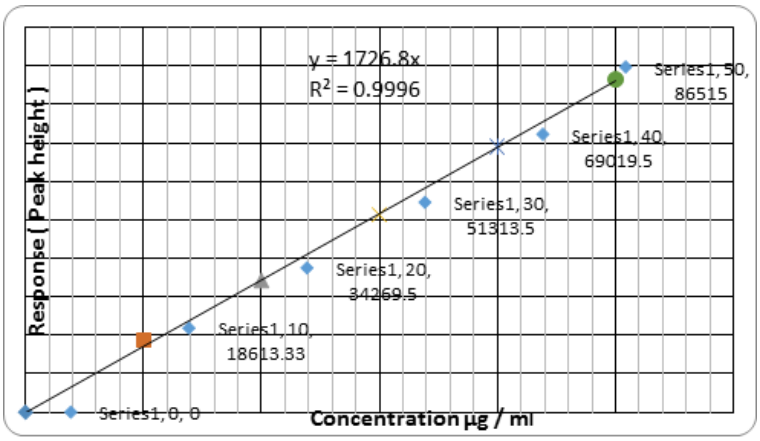

Fig.3.Peak height values versus concentrations of pyridoxine hydrochloride to demonstrate the Linearity

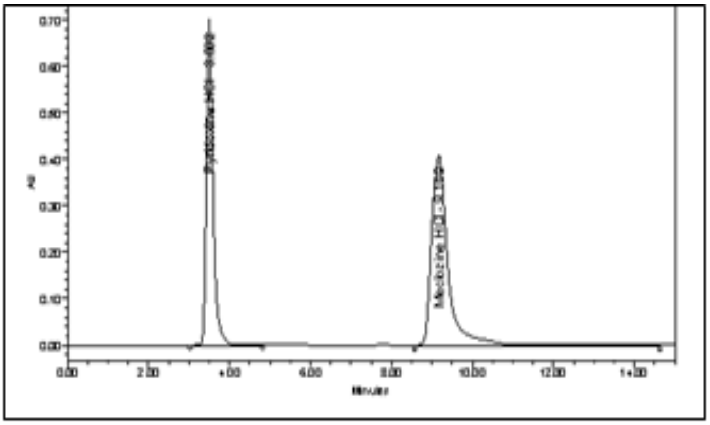

Figure.4.HPLC chromatogram test illustrate selectivity of developed validated method for combination of pyridoxine HCl (peak 1) & meclozine HCl (peak 2)

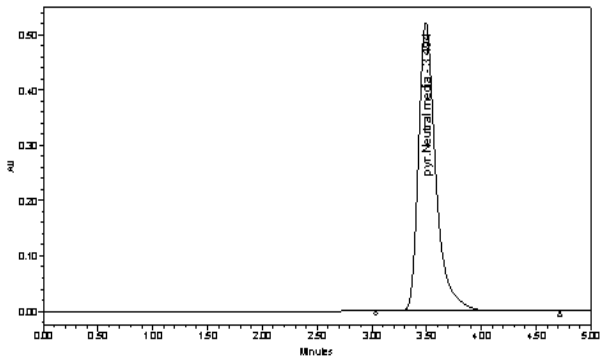

Figure.5.HPLC chromatogram test illustrate ability of developed validated method for determination of pyridoxine HCl submitted into neutral media

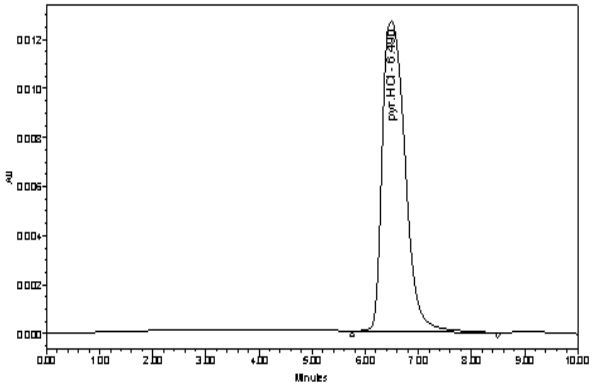

Figure.6.HPLC chromatogram test illustrate ability of developed validated method for determination of pyridoxine HCl submitted into acidic media

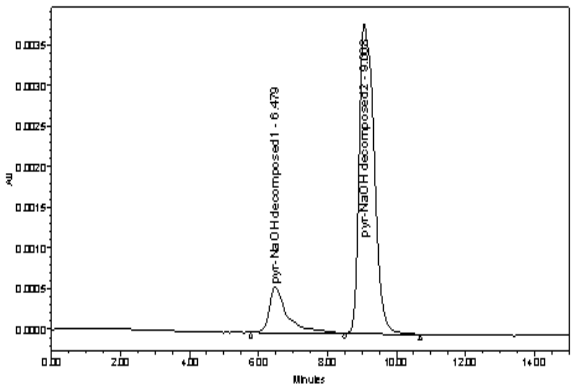

Figure.7.HPLC chromatogram test illustrate ability of developed validated method for determination of pyridoxine HCl submitted into alkaline media

4. CONCLUSION

The proposed of RP-HPLC method described enables the quantification of pyridoxine hydrochloride substance and in pharmaceutical formulation with combined dosage. The data obtained from validation studies revealed good precise, accurate and high resolution.

Developed validated method found to be simple, sensitive, accurate, saving time and less expensive, due to this data it can be used for regularly analysis of pyridoxine hydrochloride and in their combined dosage.

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