J. Appl. Biosci., 51(1): 84-93, June, 2025
doi: 10.61081/joab/51v1i108

ISSN (Print) 0975-685X
ISSN (Online) 0975-864X

ORIGINAL RESEARCH

Development and Validation of Stability Indicating RP-HPLC Method for Content Uniformity of Melatonin and Pyridoxine Hydrochloride

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ABSTRACT

Melatonin and Pyridoxine Hydrochloride combination drugs are used in the treatment of insomnia. Despite their widespread usage, the simultaneous assessment of these substances in tablet form lacks stability-indicating techniques. To warrant stability of the tablet under varied storage conditions, the study aims to establish a novel RP-HPLC technique for the estimation of Melatonin and Pyridoxine simultaneously in the formulation. Additionally, content uniformity in the tablet is also essential to ensure that patients receive a reliable and efficient dose. Hence, the formulation was also evaluated for content uniformity. Waters symmetric C18 column (3.9 x 150 mm, 5µm) was used to obtain sufficient chromatographic separation with well-defined peaks. The method used flow rate of 1ml/min, column oven temperature of 25°C, injection volume 10µl and Diode array detector at 280 nm. Mobile phase contains 20% methanol and 80% buffer (mix 670 mL water, 320 mL methanol, 10 mL glacial acetic acid and 1.4 g of 1-hexane sulphonic acid sodium salt). Method validation comprised tests for accuracy, precision, robustness, specificity, linearity and stability. Method was linear ($r > 0.999$), accurate (recovery: 98.0% - 102.0%), and precise (%RSD < 2%). Analyte stability was observed for upto 6-months in accelerated stability conditions, intermediate stability conditions and long-term stability conditions. Content uniformity tests showed that drug contents are within acceptable range.

KEY WORDS: Melatonin, Pyridoxine Hydrochloride, Method development, Method Validation, Stability studies, Content uniformity

INTRODUCTION

Melatonin, a neurohormone is produced and released in the pineal gland in the brain. Also known as N-acetyl 5-methoxytryptamine, it controls and alters the body's circadian rhythm and sleep cycle. It is hindered by light and enhanced by darkness (Bergum *et al.,* 2007; Bliesner *et al.,* 2006). *In vitro* and animal model investigations on melatonin have demonstrated its potent anti-free radical, antioxidant, and cytoprotective (Brzezinski, 1997, 2005; Chauhan *et al.,* 2021) properties. Hence melatonin is used in a number of conditions, including cancer, sleep problems, and jet lag. Chemically, it is N-[2-(5-methoxy-1H-indol-3-yl) ethylacetamide (Fig.1) having molecular weight 232.12 and is freely soluble in water (Herxheimer *et al.,* 2002).

Among the B complex vitamins, pyridoxine, or vitamin B6, is particularly notable due to its multiple functions related to health and wellness. Vitamin B6 exists in three different forms: pyridoxine (Fig. 1), found in plant products; pyridoxal; and pyridoxamine, found in animal tissues (Hudson-Curtis *et al.,* 2016; Jadav *et al.,* 2021; Japanese Pharmacopoeia XV., 2007). Pyridoxine hydrochloride (Pyridoxine HCl) is the most often utilized form of vitamin B6 in supplements and is also the most affordable to produce on a commercial scale (Kommanaboyina *et al.,* 1999). In the human body, pyridoxal phosphate, or pyridoxine hydrochloride, is produced by the pyridoxal kinase enzyme and functions as a coenzyme in over 100 enzyme-dependent reactions related to the metabolism of lipids, carbohydrates, and amino acids. It assists in minimizing the effects brought

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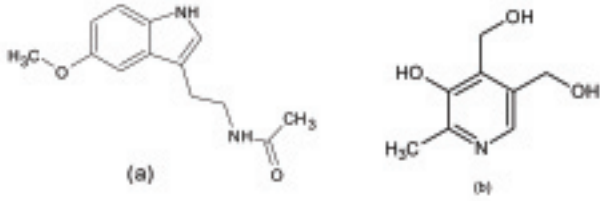

Fig. 1: Structure of Melatonin (a) and Pyridoxine Hydrochloride (b)

on by aging, diabetes, and neurological illnesses. Pyridoxine hydrochloride is available in the form of white powder of molecular mass 205.64 and is soluble in water. It is stable to heat, as well as in acidic media but unstable in light and alkaline media.

The study aimed to develop a stability indicating RP-HPLC method for the simultaneous estimation of Melatonin and pyridoxine as it is necessary to assess the stability of the tablet in different storage conditions. Moreover, drug consistency is essential to ensure patients receive a reliable and effective dose each time they use the medication. Therefore, content uniformity of the dosage form needs to be determined to ensure that different batches of the drug product meet the same quality standards. Hence, there is a need to develop novel procedures simultaneously estimating the two drugs to determine tablet content uniformity. In the pharmaceutical sector, analytical techniques are frequently employed for the quantitative and qualitative evaluation of the sample under investigation (Marz, 1999). Method development and validation techniques are employed to guarantee the identity, efficacy, purity, and functionality of pharmaceutical products, as well as confirming the stability of developed method. Separation and quantification of primary active substance, impurities, accessible synthetic intermediary substances, and any degradants are the main objectives of HPLC technique (Mayer, 1997; Merrill, 1987; Method development of analytical techniques 1999; Monika & Saranjit, 2002).

Validation is the process of determining whether an analytical procedure fulfils the requirements for its intended usage. Once the method has been developed, the validation is carried out and parameters are fixed (Panda *et al.,* 2013). Then the validated method is applied for carrying out the analysis of the regular batches. All analytical techniques have to be verified according to the International Council for Harmonisation (ICH) Guidelines-Q2 (R2), United States Pharmacopeia (USP) and other suitable regulatory guidelines (Reiter *et al.,* 2003). Stability studies are significant components in formulation development since they assure the safety, effectiveness, and quality of drugs. Drug products may lose quality due to the storage conditions such as humidity, light and temperature. These studies are intended to test the quality of pharmacological products under various situations. A pharmaceutical product's stability qualities include shelf life, storage conditions, and proper packaging. Its fundamental purpose is to guarantee that the drug product keeps its desired physical, chemical, medicinal, and microbiological qualities during its shelf life (Panda *et al.,* 2013; Saini *et al*., 2021; Sangeeta *et al.*, 2014; Sankar, 2006) . Content uniformity is one of important parameter measured in pharmaceutical industry which ensure that all drug substances within a batch contains the required amount of active pharmaceutical ingredient (API). Measuring this parameter is very important for maintaining quality, efficacy and safety of the drugs especially for low-dose formulations. Because in these type of formulations even small changes may lead to significant clinical implications (Sethi, 1996; Tan *et al.,* 2002; Toney, 2005).

MATERIALS AND METHODS

Chemicals and Reagents

Melatonin and Pyridoxine Hydrochloride are used as working standards with purity of 100% and 99%. 1-Hexane sulphonic acid obtained from Changshu Hong sheng (HPLC grade), Glacial acetic acid obtained from Honeywell (AR grade), Acetonitrile obtained from Advent (HPLC grade), Methanol obtained from Qualigens (HPLC grade) and HPLC grade water.

Instrumentation

HPLC system equipped with diode array detector-Agilent. Water symmetry C-18 column column of dimensions 3.9 X 150 mm and a particle size of 5µm is used.

Mobile phase preparation

Mix 670 mL water, 320 mL methanol and 10mL Glacial acetic acid. Dissolve 1.4 g of 1-Hexane sulphonic acid and filter through a membrane filer of 0.45 µ. Mix 800mL of above solution and 200 mL of methanol followed by sonication for 10 minutes.

Preparation of Diluent

Mix 750 mL of water and 250 mL acetonitrile and sonicate the solution for 10 minutes.

Preparation of Standard solution

Weigh and transfer accurately about 30 mg of Melatonin and 100 mg of Pyridoxine Hydrochloride into a

clean dry volumetric flask of 100 mL capacity. Add 70 mL diluent and sonicate in order to dissolve. Make up the volume upto the 100 mL mark with the diluent and mix well. Pipette out 5 mL of this solution into a 50mL flask, make up the volume with the diluent, and mix well.

Preparation of sample solution

Weigh 10 tablets, crush it and transfer into a 200mL clean, dry volumetric flask. Add 150 mL diluent and sonicate for 30 min. Cool, make the volume upto 200 mL mark with diluent and mix the contents. Centrifuge the contents at 3000rpm for 10min. Take 10 mL of the supernatant and dilute to 50 mL with diluent. Mix well.

Method development

Mobile phase selection depends on the physical-chemical properties of the compound to be analysed and the type of compound. Mobile phase is selected based on USP. The pH of the mobile phase plays an important role in the retention and separation of the multiple components. Diode array detector is used for the detection of components at 280 nm. Flow rate (1 mL/min) and Injection volume (10 µL) are selected based on USP.

Validation parameters

Specificity : Specificity is the capacity to exactly estimate the analyte in the presence of other substances that may also be present in the formulation for example excipients, contaminants, degradation products and the matrix. It shall evaluate as interference against Blank Solution, Placebo Solution, Standard Solution and Sample solution.

Linearity : Linearity is the ability of the analytical technique to obtain results directly proportional to the concentration of the sample. Different standard solutions are prepared by quantitative dilutions of standard stock solutions of Melatonin and Pyridoxine to obtain solutions in the range of 50% to 200%. Linearity shall be established over a minimum of 6 points.

Preparation of stock solution for linearity: 2.5, 3.70, 5.0, 6.30, 7.50, 10 mL of standard solutions taken in separate volumetric flask and make it up to 50mL to get 50%, 75%, 100%, 125%, 150% and 200%.

Accuracy: Accuracy of an analytical technique should be established across its range by estimating the concentration at different levels in triplicate preparations, i.e., three concentrations in three replicates. Accuracy is determined by spiking the Melatonin and Pyridoxine to the placebo solution (which contain all the excipients but no active ingredients). These samples are prepared at concentration levels of 50%, 100%, and 200% of the target concentration.

Precision : Precision of analysis is the degree of closeness between measurements obtained from consecutive samplings of the analyte under a given set of conditions.

System Precision: It is checked by using a standard to ensure that the instrument is working well. Area under the curve is measured for duplicate injections and % RSD is calculated.

Method precision: It is carried out analysing minimum 6 determinations of the sample at 100% of the test concentration.

Intermediate Precision: Ruggedeness of the procedure measures its capacity to remain unaffected by small changes such as intra-day variations, instrumental variations, analyst variations, etc.

Range

Range of an analytical technique is the interval between the upper and lower levels of the analyte that can be determined with a suitable level of accuracy, linearity and precision.

Robustness

Robustness is a measure of the method to remain unaffected by small, deliberate variations in the method parameters, and it indicates the reliability of the method for regular usage. Study was performed by altering the flow rate and the wavelength.

Stability studies

Stability of the tablets was assessed by storing the tablets at three different conditions:

Accelerated: 40°C ± 2°C/ 75% ± 5% RH (Relative Humidity)

Intermediate: 30°C ± 2°C/ 75% ±5% RH

Long term: 25°C ± 2°C/ 60% ± 5% RH

At predefined intervals (initial, 1-month, 2-months, 3-months and 6-months) samples were evaluated for:

Physical Description: Any changes in colour or appearance.

Disintegration Time: Time taken for the tablet to break down into smaller fragments.

Assay: Concentration of melatonin and pyridoxine in the tablets**.**

Content Uniformity

Sample preparation: Randomly select 10 tablets from a batch & accurately weigh each tablet. Finely crush each tablet separately using a mortar and pestle then transfer

the powder of each crushed tablet to separate 200 mL volumetric flask and makeup the volume with diluent. Dilute 10 mL of the supernatant to 50 mL with diluent and mix well. Filter the solution through a 0.45µ nylon membrane filter.

RESULTS AND DISCUSSION

Method development

In finalized method with the given chromatographic conditions (Table 1), both peak shape and resolution were good. Retention time of Pyridoxine HCl and Melatonin was found to be 2.006 and 3.600 minutes. Tailing factor for Pyridoxine HCl and Melatonin was found to be 1.4 and 1.2. Theoretical plates were more than 2000 for both the drugs (Fig. 2).

Table 1: Chromatographic conditions

Parameter	Conditions
Column	Water symmetry C18 (3.9X150 µm) 5µm
Pump mode	Isocratic
Flow rate	1 mL/min
Detector	DAD (280 nm)
Injection volume	10µL
Column temperature	Ambient
Run time	8 min

Table 2: Results for specifity

Name of the solution	Retention time
Blank	0
Placebo	0
Melatonin standard	3.598
Pyridoxine HCl standard	2.012
Melatonin sample	3.606
Pyridoxine HCl sample	2.006

Table 3: Linearity results of Melatonin and Pyridoxine HCl

Results	Melatonin	Pyridoxine HCl
Correlation Coefficient (r)	0.99991	0.99986
Regression Coefficient (R^2)	0.99982	0.9997
Slope	14.723	15.502
Y-Intercept	5.204	26.563
% deviation of Y-intercept	1.2	1.7

Table 4: Accuracy results of Melatonin and Pyridoxine HCl

Drug	%Sample Concentration	Mean % Recovery	% RSD
Melatonin	50	102.23	0.113
	100	101.4	0.151
	200	102.4	0.098
Pyridoxine HCl	50	101.73	0.0569
	100	99.53	0.0582
	200	99.93	0.0580

Table 5: Results obtained for System precision

Injection	Area of Melatonin	Area of Pyridoxine Hydrochloride
Sample 1	440.459	1556.367
Sample 2	441.114	1553.939
Sample 3	440.551	1554.123
Sample 4	441.369	1555.456
Sample 5	441.850	1554.798
Sample 6	440.963	1553.987
Mean	441.051	1556.445
%RSD	0.140%	0.114%

Table 6: Results obtained from six sample preparation for method precision

Sample Name	Area of Melatonin	Area of Pyridoxine Hydrochloride
Method precision-1	468.128	2295.070
Method precision-2	466.012	2290.003
Method precision-3	467.713	2297.288
Method precision-4	466.159	2294.852
Method precision-5	467.159	2294.456
Method precision-6	468.164	2295.741
Mean	467.222	2294.902
%RSD	0.190	0.110

Table 7: Results obtained from six sample preparations for intermediate precision

Sample Name	Area of Melatonin	Area of Pyridoxine Hydrochloride
Intermediate precision- 1	467.989	2294.561
Intermediate precision- 2	466.589	2291.874
Intermediate precision- 3	467.803	2296.100
Intermediate precision- 4	466.872	2293.753
Intermediate precision- 5	467.594	2293.589
Intermediate precision- 6	468.025	2294.321
Mean	467.145	2294.033
%RSD	0.18	0.090

Fig. 2: Chromatogram of finalized method

Fig. 3: Chromatogram of placebo

Fig. 4: Chromatogram of blank

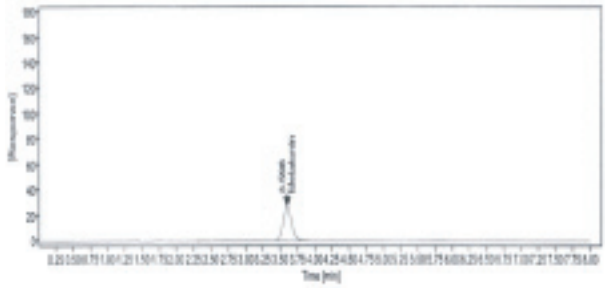

Fig. 5: Chromatogram of standard Melatonin

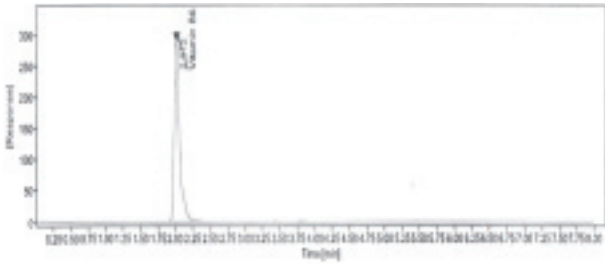

Fig. 6: Chromatogram of standard Pyridoxine HCl

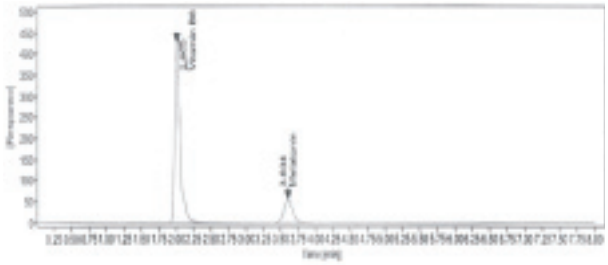

Fig. 7: Chromatogram of sample solution

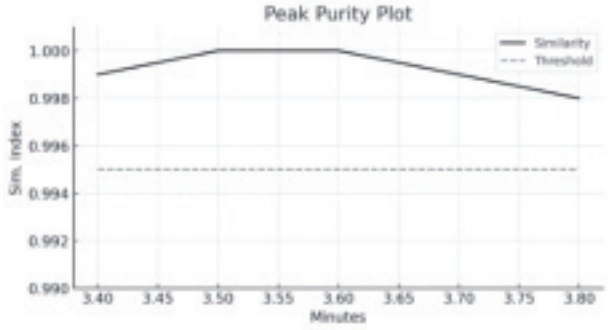

Fig. 8: Peak purity plot of Melatonin

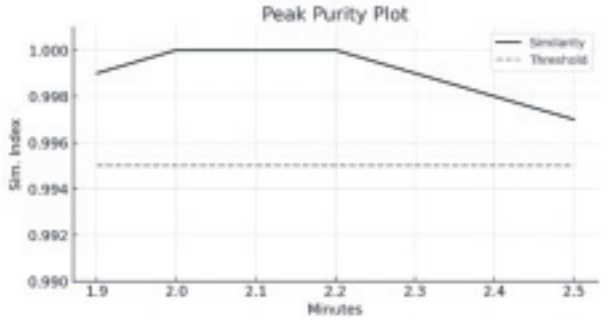

Fig. 9: Peak purity plot of Pyridoxine HCl

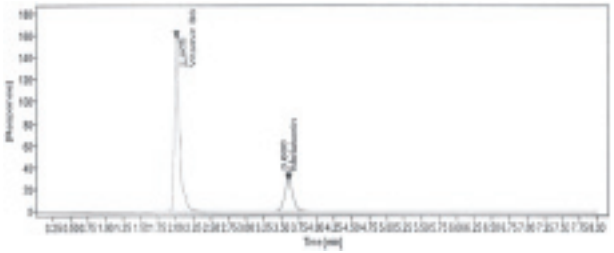

Fig. 10: Chromatogram for Standard solutions of concentration 50% and 75%

Method validation

Specificity : Chromatograms of Blank, Placebo, standard, and sample (Fig. 3-7), indicate that there is no interference at retention time of Melatonin and Pyridoxine HCl. Peak purity plots of Melatonin and Pyridoxine HCl (Fig. 8,9) in standard and sample solution indicates peaks are homogenous and has no co-eluting peaks. This further indicates the specificity of the method (Table 2).

Linearity : Standard solutions of 50% to 200% working concentration of Melatonin and Pyridoxine HCl were prepared and analysed to check linearity (Fig.10-12, Table 3), graph is given in Fig. 13.

Accuracy : Chromatograms of different concentrations of Melatonin and Pyridoxine HCl are seen in Fig. 14-16. Results are summarized in Table 4. Mean % Recovery of Melatonin and Pyridoxine HCl was found within the limit, i.e., 98-102%. Percentage relative standard deviation (RSD) of both the drugs is less than 2% for all the concentrations.

System Precision : Data shown in the Table 5 indicates that the method has acceptable level of precision Six replicate injections of standard solutions injected into the HPLC system and %RSD obtained for both Melatonin and Pyridoxine HCl was within acceptance limit, i.e., 0.140% and 0.114%.

Method Precision : Six replicate injections of sample solutions injected into the HPLC system. Results obtained by six sample preparations are summarized in Table 6 and

Table 8: Results for Range of Melatonin and Pyridoxine HCl

Parameter	Results	
	Melato-nin	Pyridoxine Hydrochloride
Linearity: Correlation coefficient(r)	0.99991	0.99986
Accuracy: % recovery		
50%	102.23%	101.73%
100%	101.4%	99.53%
200%	102.4%	99.93%
Method Precision: % RSD of result of 6 samples from method precision	0.19	0.11

Table 9: Results for Robustness with Flowrate variation

Drug	Sample name	Retention time	Assay in %	Difference in %
Melatonin	Actual	3.606	108.3	NA
	Flow minus	3.980	107.8	0.5
	Flow plus	3.245	108.1	0.2
Pyridoxine HCl	Actual	1.820	120.3	NA
	Flow minus	2.227	119.9	0.3
	Flow plus	1.819	120.2	0.1

Table 10: Robustness results with wavelength variation

Drug	Sample name	Retention time	Assay in %	Difference in %
Melatonin	Actual	3.579	107.3	NA
	Wavelength minus	3.573	107.1	0.2
	Wavelength plus	3.565	108.1	0.8
Pyridoxine HCl	Actual	2.006	119.3	NA
	Wavelength minus	2.006	118.9	0.3
	Wavelength plus	2.007	118.5	0.7

Table 11: Storage Condition: 40°C ± 2°C/ 75% ± 5%RH

Period	Description	Disintegration time	Melatonin Assay(%)	Vitamin B6 Assay(%)
Initial	White, circular, biconvex film coated tablets	12 minutes 25 seconds	98.5	116.8
1 month	White, circular, biconvex film coated tablets	11 minutes 38 seconds	99.3	118.4
2 months	Whitte, circular, biconvex film coated tablets	10 minutes 45 seconds	97.1	113.6
3 months	off-white, circular, bi-convex film coated tablets	09 minutes 50 seconds	95.6	113.9
6 months	Off-white, circular, biconvex film coated tablets	09 minutes 30 seconds	93	113.9

Table 12: Storage Condition: 30°C ± 2°C/ 75% ± 5% RH

Period	Description	Disintegration time	Melatonin Assay(%)	Vitamin B6 Assay(%)
3 months	White coloured, circular, biconvex film coated tablets	09 minutes 45 seconds	97.5	116.3
6 months	White coloured, circular, biconvex film coated tablets	09 minutes 35 seconds	94	114

Table 13: Storage Condition: 25°C± 2°C/ 60%± 5%RH

Period	Description	Disintegration time	Melatonin Assay(%)	Vitamin B6 Assay(%)
3 months	White, circular, biconvex film coated tablets	10 minutes 00 seconds	97.9	117.5
6 months	White, circular, biconvex film coated tablets	09 minutes 55 seconds	93.2	114.2

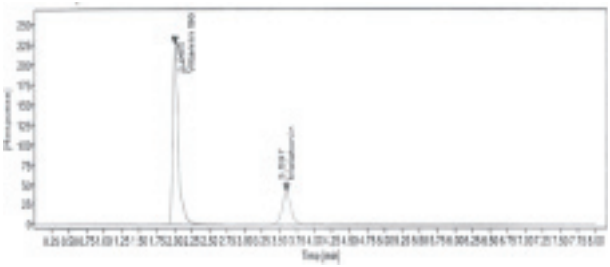

Fig. 11: Chromatogram for Standard solutions of concentration 100% and 125%

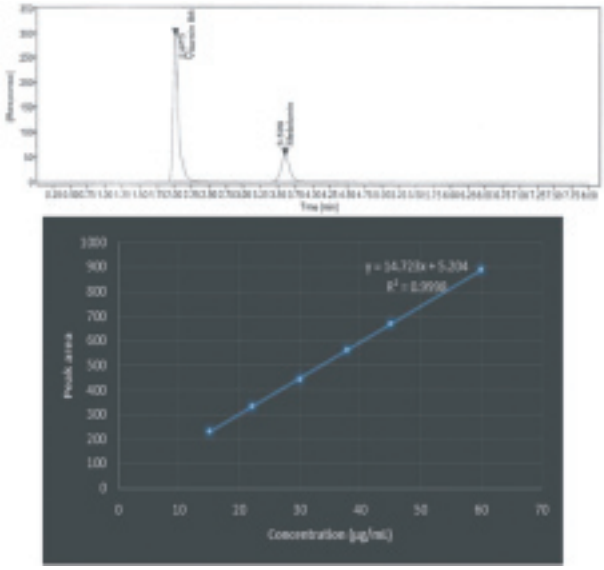

Fig. 12: Chromatograms of standard solutions of concentration 150% and 200%

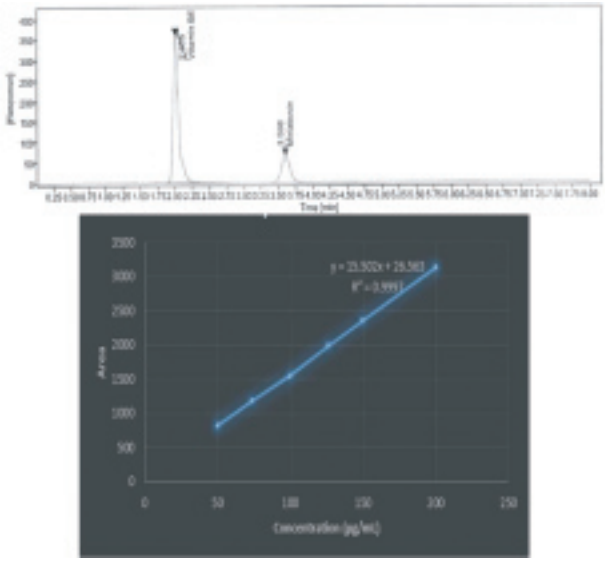

Fig. 13: Linearity plot of Melatonin and Pyridoxine HCl

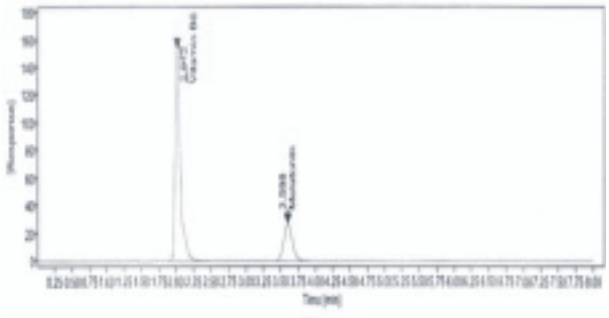

Fig. 14: Chromatogram at 50% level

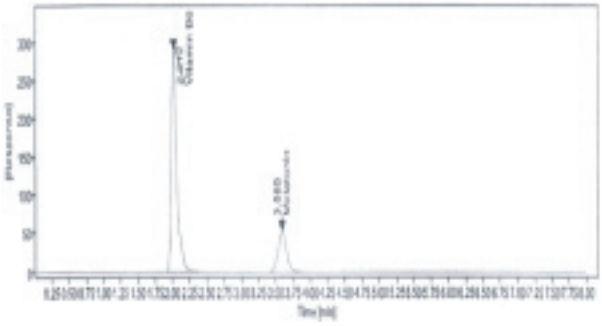

Fig. 15: Chromatogram at 100% level

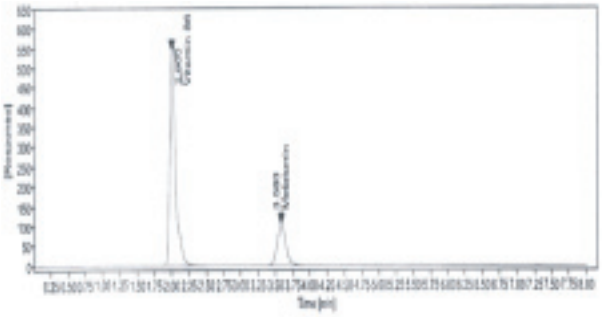

Fig. 16: Chromatogram at 200% level

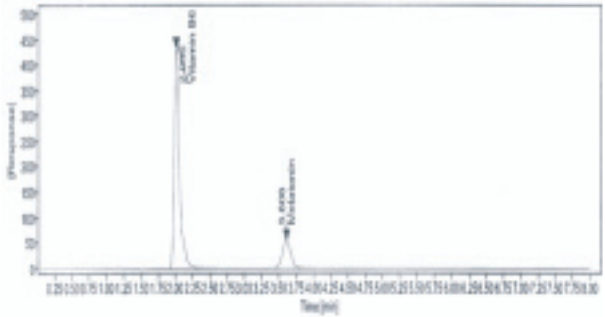

Fig. 17: Chromatogram of Unchanged Flow Rate (1 ml/min).

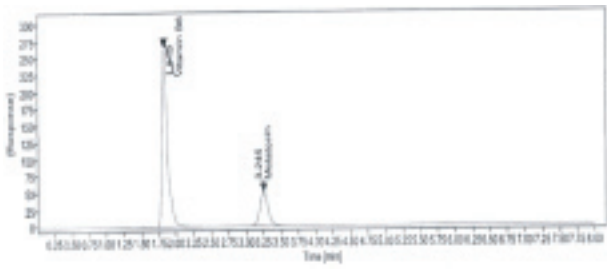

Fig. 18: Chromatogram of Flow Rate Plus (1.1 ml/min)

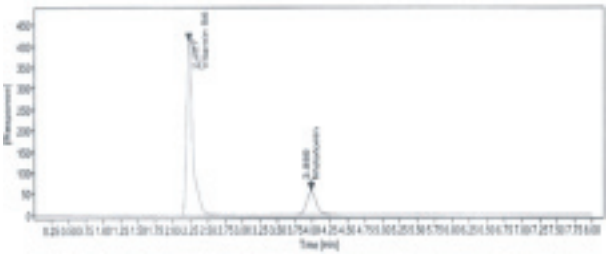

Fig. 19: Chromatogram of Flow Rate minus (0.9 ml/min)

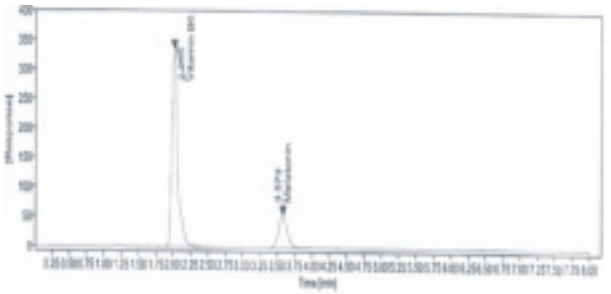

Fig. 20: Chromatogram of unchanged wavelength

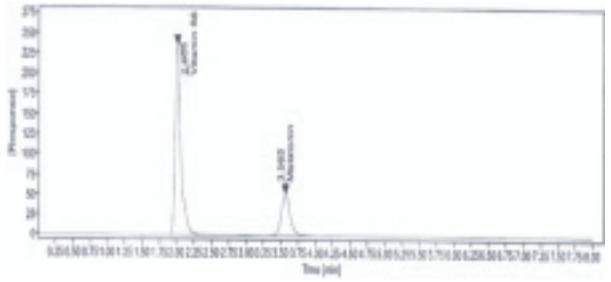

Fig. 21: Chromatogram of wavelength plus

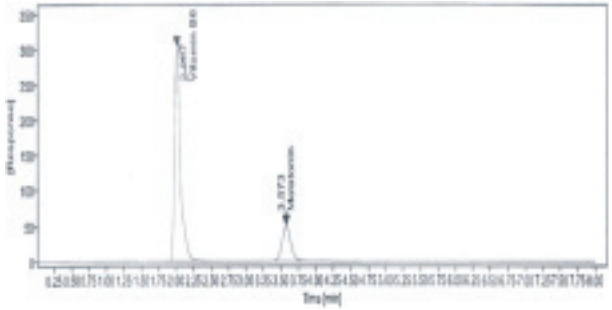

Fig. 22: Chromatogram of wavelength minus

%RSD obtained for both Melatonin and Pyridoxine HCl was within acceptance limit, i.e., 0.190% and 0.110%.

Intermediate Precision : It was verified by analysing six samples of Melatonin and Pyridoxine HCl as per the proposed methodology using different instrument and different column on different days. Results given in Table 7 indicate that %RSD of both drugs obtained from initial

Table 14: Content Uniformity data of Melatonin

Sample name	Area	% assay
Sample 1	415.635	104.4
Sample 2	412.881	103.7
Sample 3	414.260	104.1
Sample 4	421.089	105.8
Sample 5	417.412	104.9
Sample 6	406.037	102.0
Sample 7	411.552	103.4
Sample 8	418.292	105.1
Sample 9	421.813	106.0
Sample 10	417.193	104.8
Mean	104.4	
SD	1.191	
% RSD	1.1	
Minimum	102.0	
Maximum	106.0	

Table 15 Content Uniformity data of Pyridoxine HCl

Sample name	Area	% assay
Sample 1	2009.790	109.6
Sample 2	2120.508	115.6
Sample 3	2040.724	111.2
Sample 4	2081.128	113.4
Sample 5	2099.485	114.4
Sample 6	2048.010	111.3
Sample 7	2119.082	115.1
Sample 8	2026.486	110.5
Sample 9	2081.234	113.5
Sample 10	2116.626	115.6
Mean	113.1	
SD	2.214	
% RSD	2.0	
Minimum	109.6	
Maximum	115.6	

day and next day is within the acceptance limit, i.e., 0.20% and 0.10%.

Range

Table 8 summarises the results for Range of Melatonin and Pyridoxine HCl. Acceptance criteria for Linearity, is that Correlation coefficient(r) should be not less than 0.999. Accuracy of the method should be within 98-102% for all concentrations within the range.

% Relative standard deviation (RSD) results of 6 samples from method precision should be not more than 2.0%. All the criteria are met.

Robustness

Standard solutions are injected into the chromatographic system by making changes in chromatographic conditions such as ± 0.1mL/min variation in the flow rate and ± 2nm variation in wave-length. % assay difference for both Melatonin and Pyridoxine HCl are given in Table 9 and Table 10.

Data shown in the Table 9 & Table 10 indicates that the analytical method is robust to specified changes as it is within the acceptance criteria (% difference in assay results should not be more than 2% and Variation in retention time should not exceed ± 2% from the original condition)

Stability studies

Acceptance criteria for stability studies include description of the tablet should be white to off-white, circular, biconvex film coated tablets**.** Disintegration time of the tablet should not be more than 60 minutes. Percentage Assay of Melatonin, it should not be less than 90.0%. Percentage Assay of Pyridoxine HCl should not be less than 90.0% (Table 11, 12, and 13).

Content uniformity

Content uniformity of Melatonin (Table 14) and Pyridoxine HCl (Table 14) was within the acceptable range, with %RSD of 1.1 and 2.0 indicating consistency of drug within the batch.

CONCLUSION

Method development and Validation for the assay and content uniformity of Melatonin and Pyridoxine HCl is performed. Developed method is found to be accurate, reliable and precise for testing of the tablets. By addressing the essential parameters such as specificity, linearity, accuracy, precision and robustness study ensures the quality of Melatonin and Pyridoxine tablets. It successfully monitors the stability of these tablets in a variety of storage environments, assuring their quality and safety throughout their shelf life. Content uniformity results showed that the active pharmaceutical ingredients were uniformly distributed throughout all the tablets.

ACKNOWLEDGMENTS

Authors are grateful to Bioplus Life Sciences Pvt. Ltd for providing the facilities.

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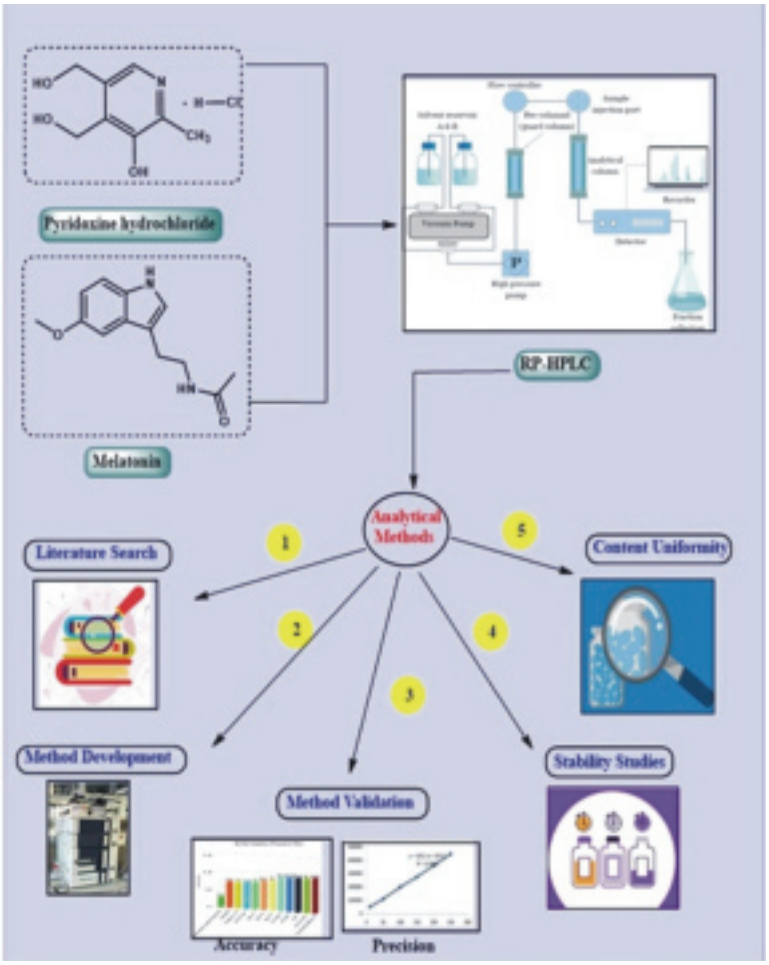

Graphical Abstract