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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF MELATONIN AND PYRIDOXINE IN BULK AND PHARMACEUTICAL DOSAGE FORM

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Abstract:
A combination of Melatonin and Pyridoxine is in called as Anti-convulsant agent. The present work deals with the RP-HPLC methods for simultaneous determination of Melatonin and Pyridoxine in pharmaceutical formation. Attempts were made to develop RP-HPLC method for simultaneous estimation of Melatonin and Pyridoxine from its formulation. For the RP HPLC method, Agilent with auto sampler Gradient System DAD Detector and C18 column with 250mm x4.6 mm i.d and 5µm particle size Acetonitrile 40%:60% water with 0.1% TEA (PH 6.2 with OPA) was used as the mobile phase for the method. The detection wavelength was 265 nm and flow rate was 1.3.3 ml/min. In the developed method, the retention time of Melatonin and Pyridoxine were found to be 2.008 min and 3.042 min. The developed method was validated according to the ICH guidelines. The linearity, precision, range, robustness was within the limits as specified by the ICH guidelines. Hence the method was found to be simple, accurate, precise, economic and reproducible.

Keyword: *Melatonin, Pyridoxine, RP-HPLC.*

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1. INTRODUCTION:

Analytical chemistry has become significantly more comprehensive of organic issues (bioanalytical chemistry) since the 1970s, when it had previously been primarily focused on inorganic or tiny organic compounds. Lasers are increasingly being employed in chemistry as probes and even to initiate and control a wide range of reactions. Analytical chemistry's claim expanded beyond scholastic material problems to forensic, environmental, industrial, and medical questions, such as in histology, in the late twentieth century. Instrumental analysis has subjugated proper laboratory chemistry. Many analytical chemists concentrate on a particular instrument type. Academics are more prone to focus on novel applications. An analytical chemist could be engaged in the identification of a chemical present in blood that raises the risk of cancer.

1.1 METHOD DEVELOPMENT

Method validation is the process of ensuring that the analytical technique utilised for a particular test is appropriate for its intended use." Method validation's repercussions can be used to moderate the quality, dependability, and consistency of analytical results; it's an important aspect of any excellent analytical procedure.

It is the process of defining an analytical requirement, and confirms that the method under consideration has performance capabilities consistent with what the application requires." [7] Use of tools that is within arrangement, operational properly and sufficiently calibrated is basic to the method validation process. The worker transport out the studies must be skilled in the analysis under study and have enough information of the method/analysis to draw conclusions from the explanation as the validation employment profits.

Method validation is a natural progression from method creation, and the two operations are frequently linked, with the validation research utilising the same methodologies and processes in the analysis as the method development.

2. DRUG PROFILE:

2.1 Melatonin

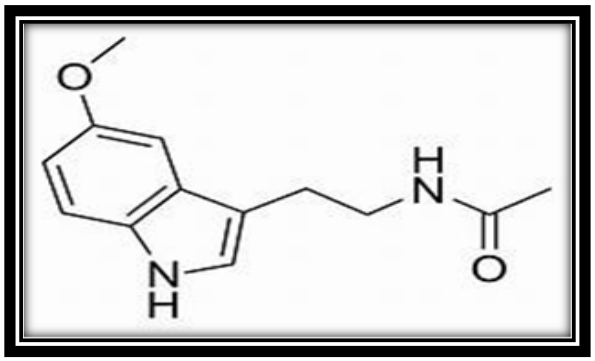

Fig. No. 1: Chemical Strcture of Melatonin.

2.1.1 Mechanism of action:

Melatonin is a derivative of tryptophan. It binds to melatonin receptor type 1A, which then acts on adenylate cylcase and the inhibition of a cAMP signal transduction pathway. Melatonin not only inhibits adenylate cyclase, but it also activates phosphilpase C. This potentiates the release of arachidonate. By binding to melatonin receptors 1 and 2, the downstream signallling cascades have various effects in the body. The melatonin receptors are G protein-coupled receptors and are expressed in various tissues of the body. There are two subtypes of the receptor in humans, melatonin receptor 1 (MT1) and melatonin receptor 2 (MT2). Melatonin and melatonin receptor agonists, on market or in clinical trials, all bind to and activate both receptor types.The binding of the agonists to the receptors has been investigated for over two decades or since 1986. It is somewhat known, but still not fully understood. When melatonin receptor agonists bind to and activate their receptors it causes numerous physiological processes. MT1 receptors are expressed in many regions of the central nervous system (CNS): suprachiasmatic nucleus of the hypothalamus (SNC), hippocampus, substantia nigra, cerebellum, central dopaminergic pathways, ventral tegmental area and nucleus accumbens. MT1 is also expressed in the retina, ovary, testis, mammary gland, coronary circulation and aorta, gallbladder, liver, kidney, skin and the immune system. MT2 receptors are expressed mainly in the CNS, also in the lung, cardiac, coronary and aortic tissue, myometrium and granulosa cells, immune cells, duodenum and adipocytes.

2.2 Pyridoxine

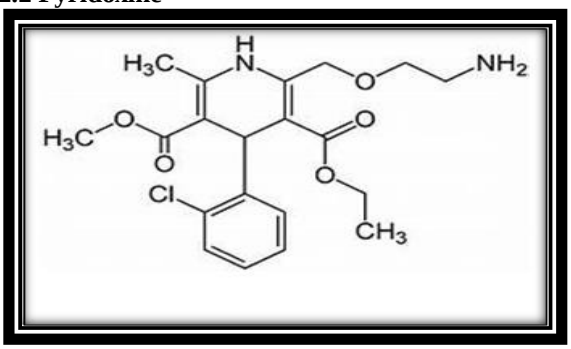

Fig. No 2: Chemical Structure of Pyridoxine

2.2.1 Mechanism of action

Vitamin B6 is the collective term for a group of three related compounds, pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM), and their phosphorylated derivatives, pyridoxine 5'-phosphate (PNP), pyridoxal 5'-phosphate (PLP) and pyridoxamine 5'-phosphate (PMP). Although all six of these compounds should technically be referred to as vitamin B6, the term vitamin B6 is commonly used interchangeably with just one of them, pyridoxine. Vitamin B6, principally in its biologically active coenzyme form pyridoxal 5'-phosphate, is involved in a wide range of biochemical reactions, including the metabolism of amino acids and glycogen, the synthesis of nucleic acids, hemoglobin, sphingomyelin and other sphingolipids, and the synthesis of the neurotransmitters serotonin, dopamine, norepinephrine and gamma-aminobutyric acid (GABA).

3. MATERIALS AND METHODS:

3.1 Selection and Procurement of Drug

3.1.1 Name of Drug

i. Melatonin
ii. Pyridoxine

Table No. 1: List of Reagents and Chemicals used

Ingredients	Grade
Melatonin	API
Pyridoxine	API
Acetic Acid	HPLC
Orthophopsphoric acid(OPA)	HPLC
Methanol	HPLC
Water	HPLC

3.2 HPLC:

3.2.1 Selection of Analytical Technique

- HPLC was selected as analytical technique for estimation of Melatonin and Pyridoxine.

Stock preparations:

- Stock I : Standard Sample Preparation

Std. Melatonin and Pyridoxine e 1:3.3 mg in 10 ml Methanol = 500:1500 µgm/ml

- Stock II : Tab solution Preparation:-

Take 52.5 mgs in 10 ml Methanol i.e= 500:1500 µgm/ml

4. EXPERIMENTAL WORK:

4.1 Chromatographic conditions:

The following chromatographic conditions were established by trial and error and were kept constant throughout the experimentation.

Table No.2: chromatographic conditions (HPLC) details used during method Development

1.	HPLC	Agilent Tech. Gradient System With Auto injector
2.	Software	Chemstation 10.1 software
3.	Column	(Agilent) C18 column (4.6mm x 250mm)
4.	Particle size packing	5 µm
5.	Stationary phase	C-18 (Agilent)
6.	Mobile Phase	Methanol : Water (0.1% Acetic Acid PH adjusted 3.5 with OPA) 65 : 35
7.	Detection Wavelength	265 nm
8.	Flow rate	1.0 ml/min
9.	Temperature	25
10.	Sample size	20 µl
11.	pH	3.5
12.	Run Time	15 min
13.	Filter paper	0.45 µm

4.2 METHOD DEVELOPMENT OF HPLC:

- **List of Mobile Phase :**

Table No.3: Selection of mobile Phase.

Sr.No.	Mobile Phase
1.	METHANOL+ Water OPA 0.1 %(80:20 % v/v) 0.7ml 265 nm
2.	METHANOL+ Water OPA 0.1%(35:65 % v/v) 1ml 265 nm
3	ACN+ Water TEA 0.1%(30:70 % v/v) 1 ml 265 nm
4	ACN+ Water TEA 0.1%(40:60 % v/v) 1 ml 265 nm
5	ACN+ Water OPA 0.1%(90:10 % v/v) 1ml 265 nm sample in methanol
6	METHANOL+ Water OPA 0.05%(60:40 % v/v) 1.ml 265 nm sample in mobile phase
7	ACN+ Water TEA 0.1%(40:60 % v/v) 1.1 ml 265 nm

4.3. Analysis of standard drugs was done by following parameters:

- Melting point
- Solubility
- UV spectra and λ_{max}
- U-HPLC chromatogram and retention time

4.4. Selection of wavelength by UV-Visible Spectrophotometry:-

4.4.1. Preparation of standard stock solution:

- **Melatonin standard stock solution : (Stock I)**

An accurately weighed quantity, 5 mg of Melatonin (MLT) was dissolved in methanol in a 10 ml volumetric flask and volume made up to 10.0 ml to produce a solution of 500 ug/ml.

- **Pyridoxine standard stock solution : (Stock II)**

An accurately weighed quantity, 15 mg of Pyridoxine (PYD) was dissolved in methanol in 10 ml volumetric flask and volume made up to 10.0 ml to produce a solution of 1500 ug/ml.

- **Preparation of Stock Standard Combination Solution :(Stock III) [MLT+PYD]**

Accurately weight and transfer 5 mg Melatonin and Pyridoxine 15 mg working standard into 10 ml volumetric flask as about diluent methanol completely and make volume up to the mark with the same solvent to get 500 ug/ml standard for Melatonin and 1500 µg/ml for Pyridoxine (stock solution) and 15 min sonicate to dissolve it and remove the unwanted gas.

4.4.2. HPLC used for chromatographic condition applies on the Preparation of standard solution:-

- **Preparation of std. Melatonin solution: (Stock I)**

From the freshly prepared standard stock solution (500 ug/ml), 0.1-0.5 ml stock solution was pipetted out in 10 ml of volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration of 5-25 ug/ml.

- **Preparation of std. Pyridoxine solution: (Stock II)**

From the freshly prepared standard stock solution (1500ug/ml), 0.1-0.5 ml stock solution was pipetted out in 10 ml of volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration 15-75 ug/ml.

- **Preparation of std. Melatonin and Pyridoxine solution :(Stock III)**

From the freshly prepared standard stock solution (500 ug/ml) Melatonin, and (1500 ug/ml) Pyridoxine , 0.1-0.5 ml stock solution was pipette out in 10 ml of volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration 5-25 ug/ml for Melatonin and Pyridoxine 15-75 ug/ml respectively.

4.3. Selection of mobile phase:

Each mobile phase was vacuum degassed and filtered through 0.45µ membrane filter. The mobile phase was allowed to equilibrate until Acetic Acid by baseline was obtained

4.4. Procedure for calibration curve of Melatonin and Pyridoxine

The mobile phase was allowed to equilibrate with stationary phase until Acetic Acid by baseline was obtained. From the freshly prepared standard stock solution, pipette out 5 mg Melatonin and 15 mg Pyridoxine in 10 ml of volumetric flask and diluted with Methanol. From it 0.1, 0.2, 0.3, 0.4 and 0.5 ml of solution were pipette out in 10 ml volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration 5,10,15,20 and 25 µg/ml of Melatonin and 15,30,45,60 and 75 µg/ml of Pyridoxine.

4.5. Study of system suitability parameters:

The system suitability is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis to be done. The test

was performed by collecting data from five replicate injections of standard solution.

4.5.1. Calibration Experiment:

- **RP-HPLC Method :**

a) Preparation of Calibration curve standard:

The above standard stock solution (500:1500µg/ml) of Melatonin and Pyridoxine was diluted with mobile phase to yield Five calibration curve (cc) standards with concentrations of 5,10,15,20 and 25 µg/ml of Melatonin and 15,30,45,60 and 75 µg/ml of Pyridoxine

4.6. Validation of method for analysis of Melatonin and Pyridoxine

- The developed method was validated as per ICH guidelines.

4.6.1 Linearity:

Linearity of an analytical method is its ability to elicit test results that are directly or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range.

Table No.4: Table of linearity for RP -HPLC Method

Concentration (µg/mL)	
Melatonin	Pyridoxine
5	15
10	30
15	45
20	60
25	75

4.6.2 Accuracy (recovery):

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Accuracy may often the expressed as percent recovery by the assay of known added amounts of analyte.

- **Accuracy**

The accuracy was determined by Melatonin and Pyridoxine (equivalent to 5 mg of Melatonin and 15 mg of Pyridoxine (80 %, 100 % and 120 % of the label claimed, respectively) to quantity equivalent to average weight of marketed tablets. This powder mixture containing 5 mg of Melatonin and 15 mg of Pyridoxine were triturated and then subjected to chromatographic analysis using the described method.

Table No. 5: Table of Accuracy for Rp-HPLC Method

Sample	Amount Added (mg)	
	Melatonin	Pyridoxine
Accuracy 80%	4	12
Accuracy 100%	5	15
Accuracy 120%	6	18

4.6.3 Repeatability:

Precision of the system was determined with the sample of RP-HPLC for. Two replicates of sample solution containing 5 mg of Melatonin and 15 mg Pyridoxine were injected and peak areas were measured and %RSD was calculated is was repeated for two times

- **Application of proposed method for analysis of Repeatability:**

Weight 5 mg of Melatonin and 15 mg Pyridoxine were weighed and transferred to 10 mL volumetric flask & diluent was added to make up the volume. Sonicated for 10 min with occasional swirling. The above solution was filtered through 0.45µm membrane filter 0.3 ml of this solution diluted upto 10 ml with diluent.

4.6.4 Precision:

Precision of an analytical method is the degree of agreement among Individual test results when the procedure is applied repeatedly to multiple Samplings of a homogenous sample. Precision of an analytical method is usually expressed as standard deviation or relative standard deviation.

4.6.4.1 Result of Intraday and Inter day Precision studies on RP-HPLC method for Melatonin and Pyridoxine

I. **Intra-day precision:**
Sample solutions containing 5 mg of Melatonin and 15 mg of Pyridoxine three different concentration (10 µg/ml, 15 µg/ml, 20µg/ml Melatonin and (30 µg/ml, 45 µg/ml, 60 µg/ml) Pyridoxine.

II. **Inter-day precision:**
Sample solutions containing 5 mg of Melatonin and 15 mg of Pyridoxine three different concentration (10 µg/ml, 15µg/ml, 20µg/ml Melatonin and (30 µg/ml, 45 µg/ml, 60 µg/ml) Pyridoxine.

4.6.5. Robustness:

The mobile phase composition was changed in (±1 ml/ min^{-1}**)** proportion of Methanol: Water (0.1%

TEA PH adjusted 6.2 with OPA) the mobile phase composition and the flow rate was (±1ml/ min^{-1}**)** and the change in detection wavelength (±1 ml/ min^{-1}**)** and the effect of the results were examined.

4.6.6 Detection Limit

Based on the S.D. of the response and the slope of calibration curve, the detection limit (DL) was calculated as,

$DL = \frac{3.3\sigma}{S}$

4.6.7 Quantitation Limit

Based on the S.D. of the response and the slope of calibration curve, the quantitation limit (QL) was calculated as,

$QL = \frac{10\sigma}{S}$

5. RESULT AND DISCUSSIONS:

5.1. Preliminary studies on Melatonin and Pyridoxine

5.1.1. Melting point

The procured reference standard of Melatonin and Pyridoxine were found to melt in the range of 117 ^{0}C and 159-162 ^{0}C respectively.

5.1.2. Solubility

The drug was found to be

- Slightly soluble in Water and ethanol, propanol.
- Pyridoxine is soluble in water, methanol.
- Insoluble in ethanol.

5.1.3. UV Spectroscopy

UV absorption of 10 µg/mL solution of Melatonin and Pyridoxine in Methanol was generated and absorbance was taken in the range of 200-400 nm.λmax of Melatonin and Pyridoxine in Methanol was found to be 236 nm and 274 nm respectively.

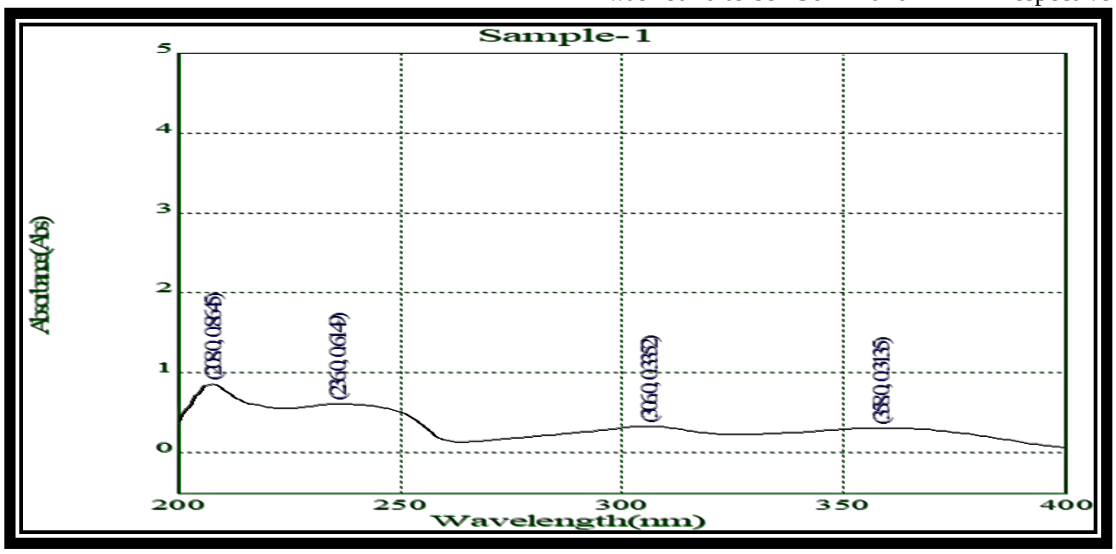

Fig.No. 3 Uv spectrum of Melatonin

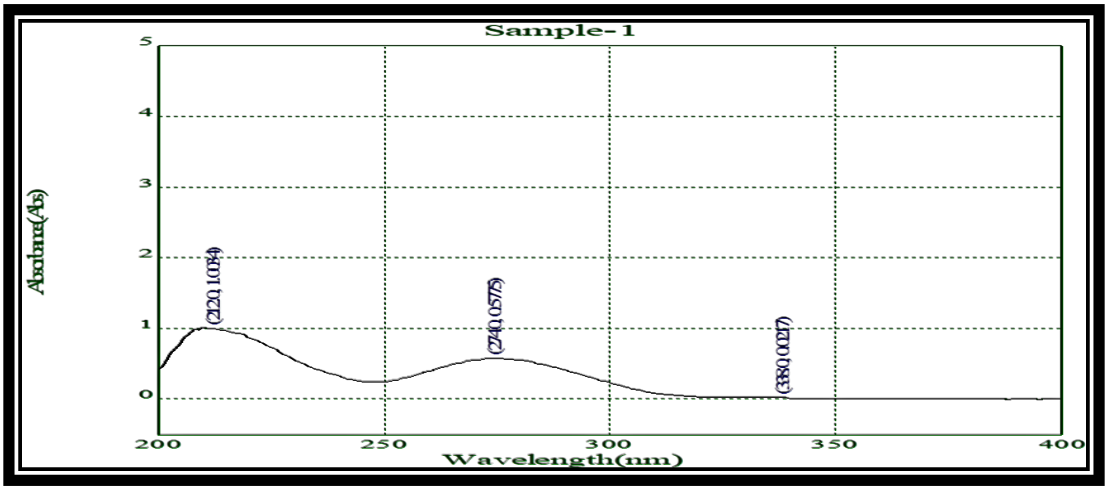

Fig. No 4. Uv spectrum of Pyridoxine

5.2 Studies on the chromatographic behavior of Melatonin and Pyridoxine

Table. No 6: Chromatographic behavior of Melatonin and Pyridoxine mobile phase of various compositions

Sr No.	Mobile Phase	Retention Time (min)		Remark
		MLT	PYD	
1.	METHANOL+ Water OPA 0.1 %(80:20 % v/v) 0.7ml 265 nm	3.402	5.400	No Sharpe peak
2	METHANOL+ Water OPA 0.1%(35:65 % v/v) 1ml 265 nm	2.471	4.115	No Sharpe peak
3	ACN+ Water TEA 0.1%(30:70 % v/v) 1 ml 265 nm	2.311	3.489	No Sharpe peak
4	ACN+ Water TEA 0.1%(40:60 % v/v) 1 ml 265 nm	2.051	3.112	No Sharpe peak

a) Chromatogram of Trial 1:

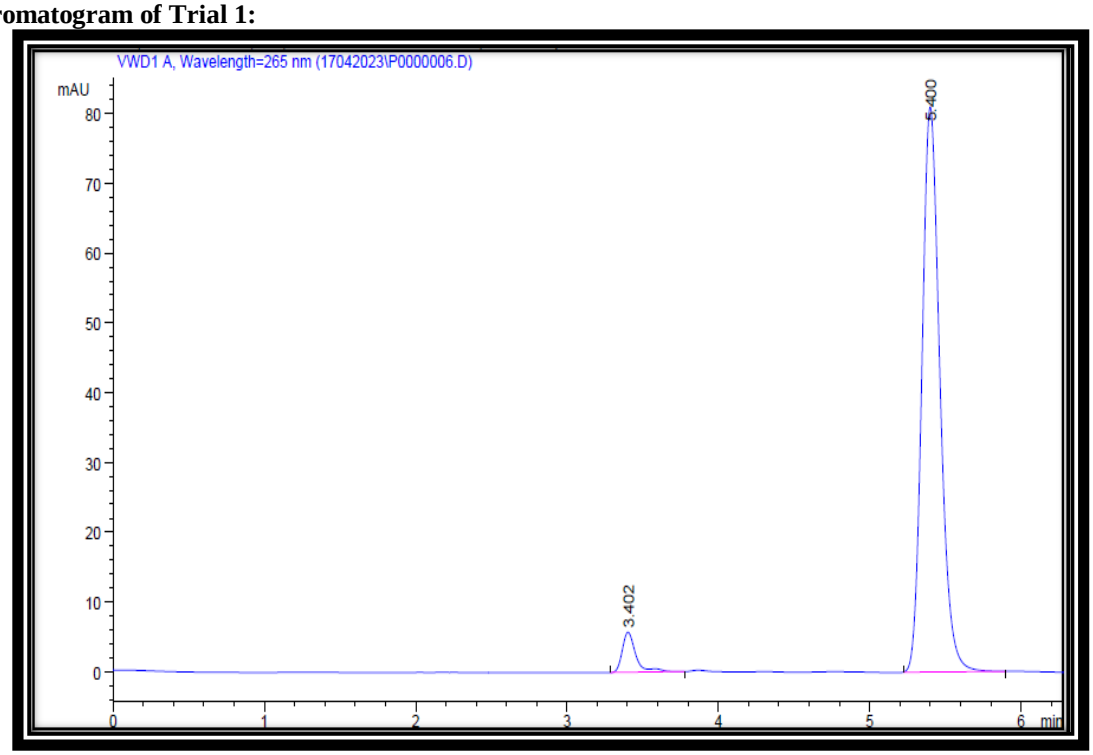

Fig. No 5: Representative Chromatogram of Melatonin and Pyridoxine using MLT+PYD 80 % Methanol+ 20 0.1 % OPA -265 nm- 0.7ML- 20 MCG as mobile phase, showing the no retention time.

Table No. 7: Chromatogram result of Melatonin and Pyridoxine

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	3.402	36.2964	8641	0.63	-
2	5.400	667.7863	10685	0.78	11.22

b) Chromatogram of Trial 2:

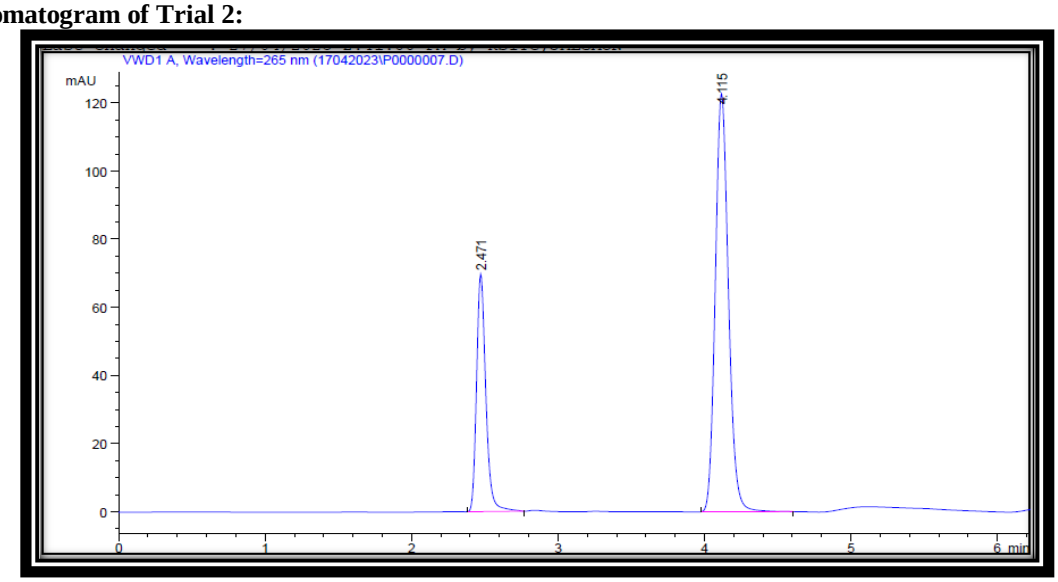

Fig. No 6:Representative Chromatogram of Melatonin and Pyridoxine using MLT+PYD 35 % Methanol+ 65 (0.1 % OPA)-265 nm- 1ML-20 mcg mobile phase.

Table No. 8: Chromatogram result of Melatonin and Pyridoxine.

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	2.471	306.58282	8241	0.73	0.0000
2	4.115	767.1330	10656	0.83	1.67

c) Chromatogram of Trial 3:

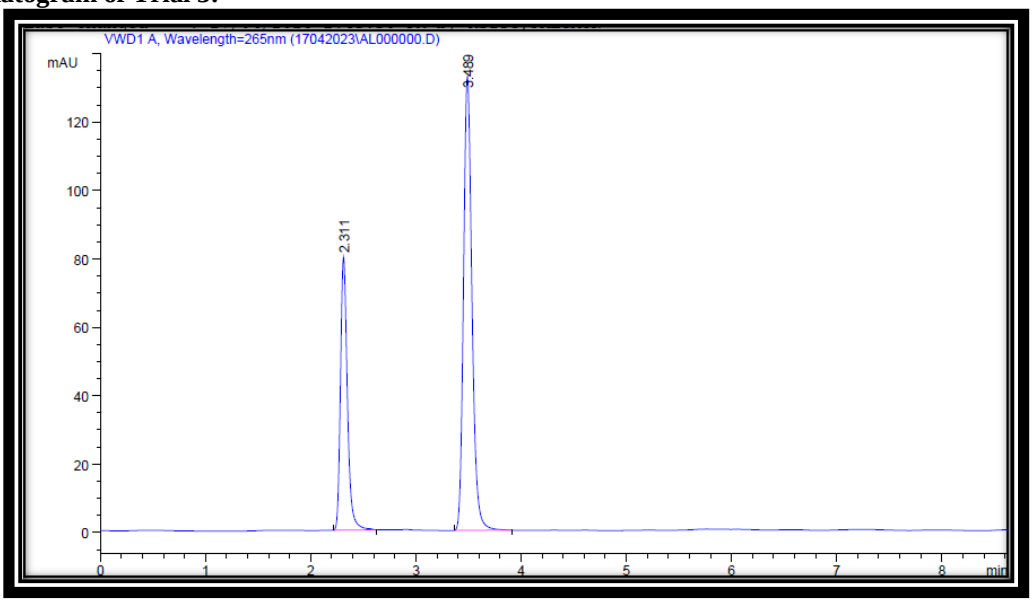

Fig. No 7: Representative Chromatogram of Melatonin and Pyridoxine using MLT+PYD 30 % ACN+ 70 (0.1 % TEA PH 6.2 with OPA)-265 nm- 1.0ML

Table No. 9: Chromatogram result of Melatonin and Pyridoxine

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	2.311	363.84747	6596	0.70	0.0000
2	3.489	742.2844	9904	0.76	9.26

5.3 Calibration experiment

- **RP-HPLC Method :**

Table No 10: Linearity data for Melatonin

Method	Conc µg/ml	Peak area(µV.sec)		Average peak area (µV.sec)	S.D. of Peak Area	% RSD of Peak Area
		1	2			
RP-HPLC Method	5	224.4567	223.3868	223.9218	0.76	0.34
	10	362.4423	362.32	362.3812	0.09	0.02
	15	479.1152	474.2246	476.6699	3.46	0.73
	20	596.3431	597.8256	597.0844	1.05	0.18
	25	723.7741	729.5788	726.6765	4.10	0.56
	Equation		y = 24.09X+120.3			
	R^2		0.999			

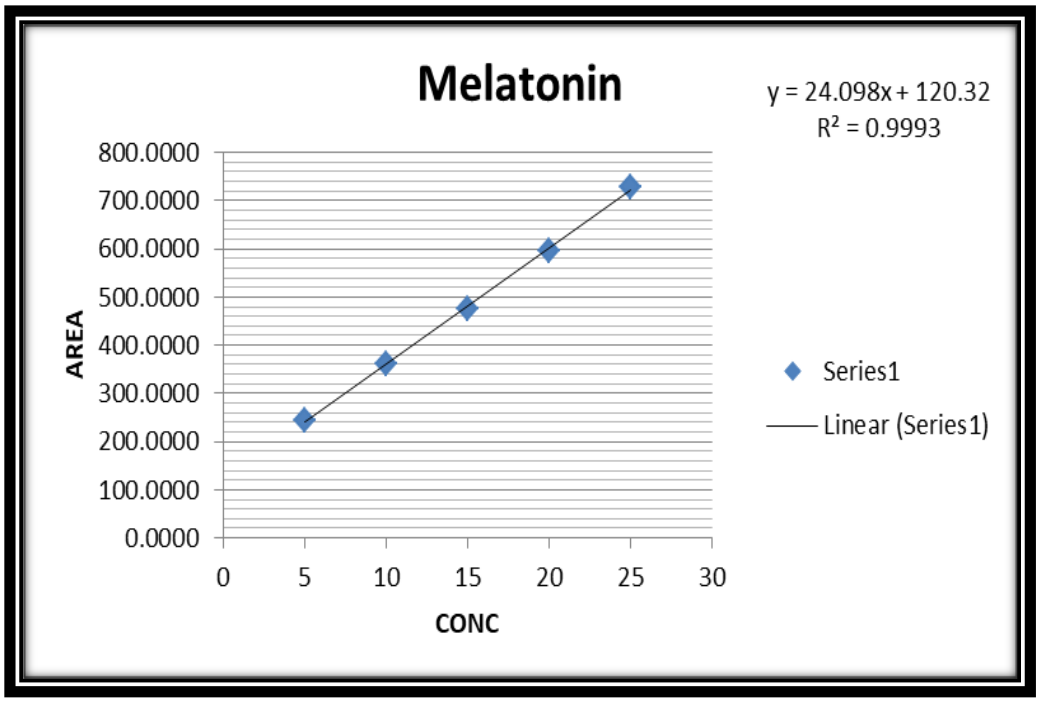

Fig.No.8: Calibration curve of Melatonin

The RP-HPLC Method for respective linear equation for Melatonin was y = 24.09X+120.3 where x is the concentration and y is area of peak. The correlation coefficient was 0.999. The calibration curve of Melatonin is depicted in Fig 9.

Table No 11: Linearity data for Pyridoxine

Method	Conc µg/ml	Peak area(µV.sec)		Average peak area (µV.sec)	S.D. of Peak Area	% RSD of Peak Area
		1	2			
RP-HPLC Method	15	268.1256	269.9567	269.0412	1.2948	0.4813
	30	516.4201	517.5354	516.9778	0.7886	0.1525
	45	777.7319	775.9973	776.8646	1.2265	0.1579
	60	1039.0156	1040.3060	1039.6608	0.9125	0.0878
	75	1307.3835	1309.3336	1308.3586	1.3789	0.1054
	Equation		y = 17.34 x + 1.785			
	R^2		0.999			

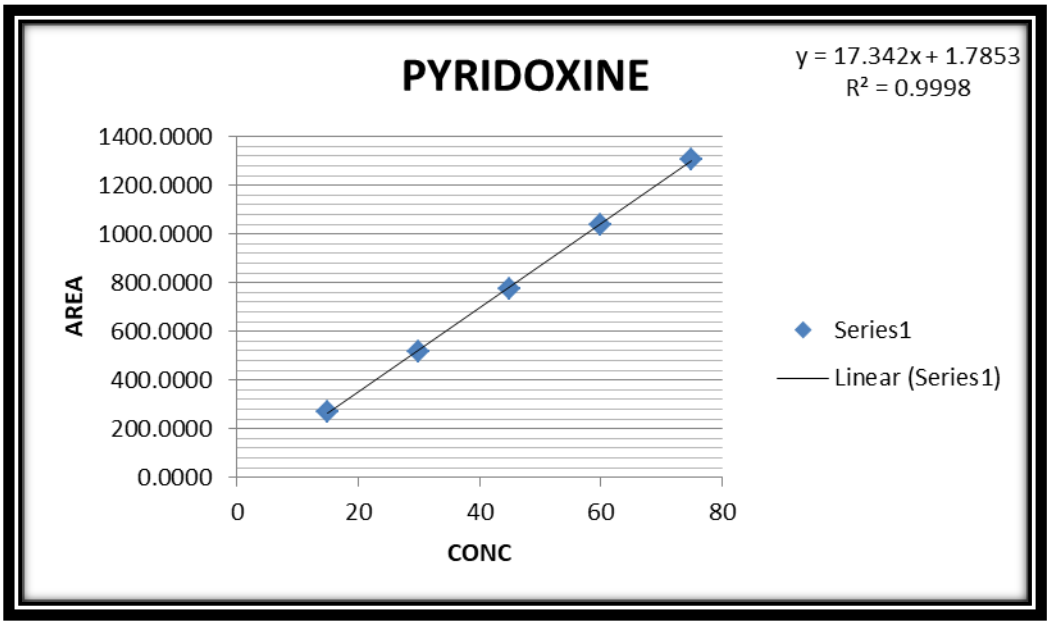

Fig.No.9: Calibration curve of Pyridoxine

The RP-HPLC method for respective linear equation for Pyridoxine was y = 17.34 x+1.785 where x is the concentration and y is area of peak. The correlation coefficient was 0.999. The calibration curve of Pyridoxine is depicted in Fig 10.

5.4 Analytical of Method Validation:

1. Linearity:

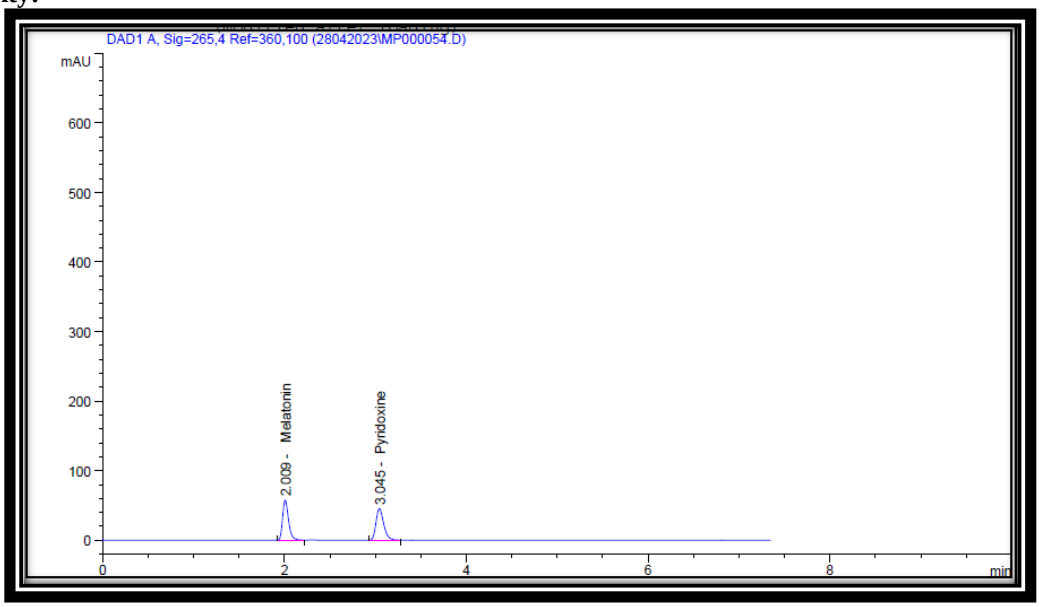

Fig.No.10. Chromatogram of linearity 5+15 mcg-01

Table No 12: Chromatogram of linearity 5 +15 mcg-01

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	2.009	224.45671	4999	0.72	0.0000
2	3.045	268.12560	6781	0.72	7.78

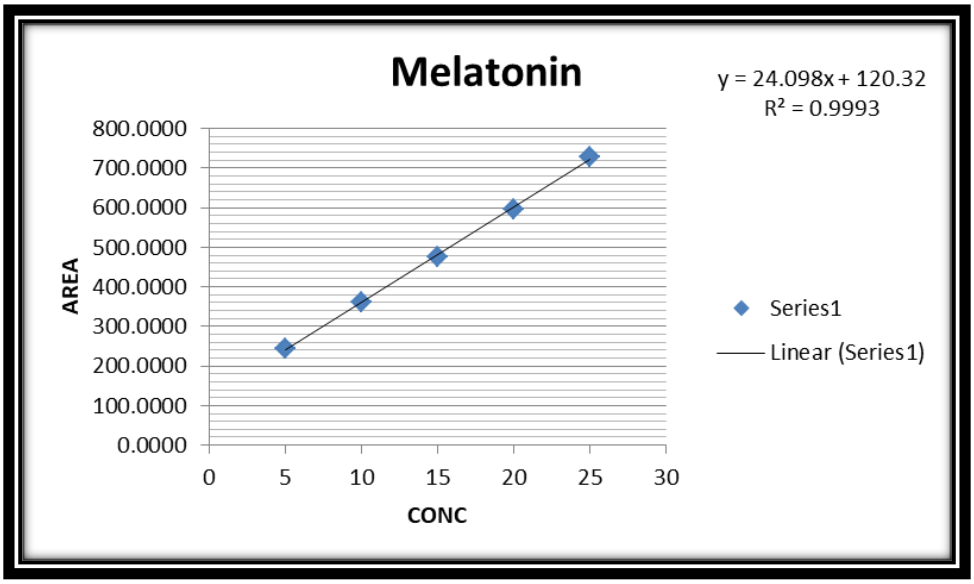

Fig.No.11. Calibration curve of HPLC method

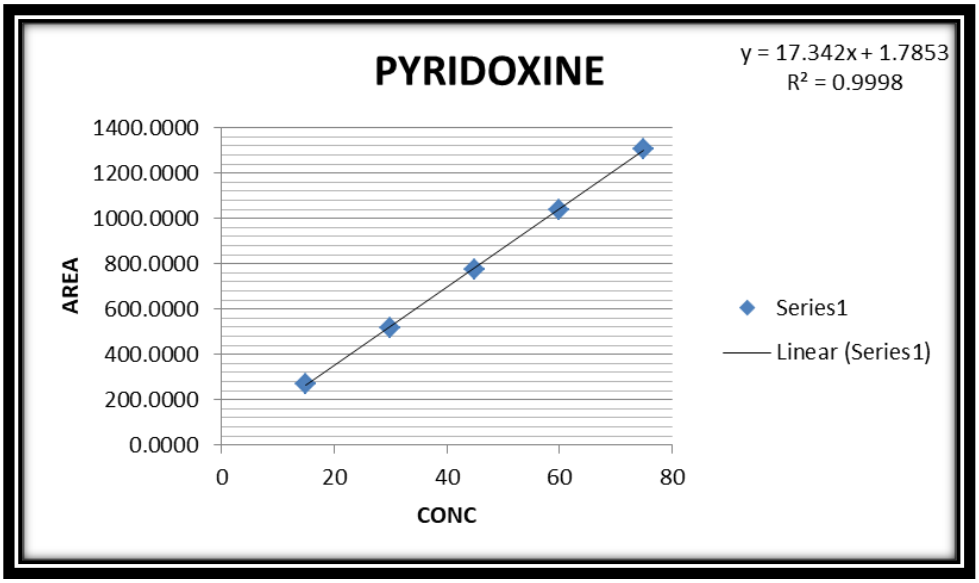

Fig No. 12: Calibration graph of Pyridoxine for HPLC method

6. SUMMARY AND CONCLUSION:

Simple, rapid, accurate and precise RP-HPLC methods have been developed and validated for the routine analysis of Melatonin and Pyridoxine in API and formulation. Both methods are suitable for the simultaneous determination of Melatonin and Pyridoxine in multi-component formulations without interference of each other. The developed methods are recommended for routine and quality control analysis of the investigated drugs in two component pharmaceutical preparations. The amount found from the proposed methods was in good agreement with the label claim of the formulation. Also the value of standard deviation and coefficient of variation calculated were satisfactorily low, indicating the suitability of the proposed methods for the routine estimation of tablet dosage forms.

7. ACKNOWLEDGEMENT

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8. CONFLICTS OF INTEREST

Authors have no conflicts of interest to declare.

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