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SIMULTANEOUS DETERMINATION BY RP-HPLC OF PYRIDOXINE HYDROCHLORIDE, DI METHIONINE AND NICOTINAMIDE IN TABLETS

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ABSTRACT

A simple, specific, accurate, precise stability indicating reversed-phase high-performance liquid chromatographic (RP-HPLC) method was developed and validated for the simultaneous determination of Pyridoxine hydrochloride (PYH) Dl methionine (DMN) and Nicotinamide (NCM). An isocratic separation of PYH,DMN and NCM were achieved on C 18, 250 × 4.6 mm ID, 5 µm particle size columns at column oven temperature 37°C with a flow rate of 0.5 mL min−1 and using a diode array detector to monitor the detection at 254 nm. The mobile phase consisted of buffer : acetonitrile : trifluoroacetic acid at a ratio of 30 : 70 : 0.1 (v/v). The retention times of PYH DMN and NCM was found to be 5.25 and 10.14 min, respectively. Suitability, specificity, linearity, accuracy, precision, stability, and sensitivity of this method for the quantitative determination of the drugs were proved by validation in accordance with the requirements laid down by International Conference on Harmonization (ICH) Q2 (R1) guidelines. The proposed method is reliable and robust and can be used as quality control tool for the estimation of these drugs in combined pharmaceutical solid dosage forms.

KEYWORD: RP-HPLC, Pyridoxine hydrochloride, Dl methionine, Nicotinamide.

INTRODUCTION - DRUGS

1. Pyridoxine Hydrochloride

Pyridoxine hydrochloride is the hydrochloride salt of pyridoxine, a water-soluble vitamin belonging to the vitamin B-complex (Vitamin B6) group. It plays a vital role in amino acid

metabolism, neurotransmitter synthesis, hemoglobin formation, and lipid metabolism. Pyridoxine acts as a precursor of the active coenzyme pyridoxal-5-phosphate, which is involved in numerous enzymatic reactions in the body. Deficiency of pyridoxine may lead to conditions such as peripheral neuropathy, anemia, dermatitis, irritability, and convulsions. Therapeutically, pyridoxine hydrochloride is used in the treatment and prevention of vitamin B6 deficiency, drug-induced neuropathy (e.g., due to isoniazid), pregnancy-related nausea, and certain metabolic disorders.

2. DL-Methionine

DL-Methionine is a synthetic racemic mixture of D- and L-isomers of methionine, an essential sulfur-containing amino acid. Methionine is required for protein synthesis, transmethylation reactions, and detoxification processes in the liver. It serves as a precursor for biologically important compounds such as cysteine, taurine, glutathione, and S-adenosylmethionine (SAM). DL-Methionine is widely used as a nutritional supplement and as a lipotropic agent to prevent fatty liver. It is also used to acidify urine, thereby helping in the management of certain urinary tract infections and kidney stone prevention. Methionine deficiency can result in liver damage, poor growth, and weakness.

3. Nicotinamide

Nicotinamide, also known as niacinamide, is the amide form of niacin and belongs to the vitamin B3 group. It is a vital component of the coenzymes nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$), which are essential for cellular respiration, energy production, and redox reactions. Unlike nicotinic acid, nicotinamide does not cause vasodilation or flushing. Deficiency of nicotinamide leads to pellagra, characterized by dermatitis, diarrhea, and dementia. Clinically, nicotinamide is used in the treatment and prevention of vitamin B3 deficiency, skin disorders, and as a nutritional supplement.

DRUG PROFILE

Pyridoxine Hydrochloride

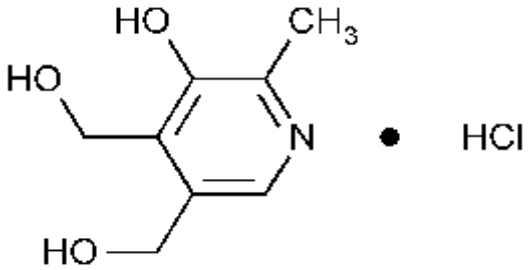

Figure 1.1: Molecular Structure of Pyridoxine Hydrochloride.

IUPAC Name : **4,5-bis(hydroxymethyl)-2-methylpyridin-3-ol;hydrochloride**

Molecular formula : $C_8 H_{11}NO_3HCl$

Molecular weight : 205.64 g/mol

Pharmacokinetic Data

Bioavailability : 75 To 95%

Protein binding : 60%

Metabolism : Hepatic

Excretion : 75% Renal, 21-25% Feces

DI Methionine

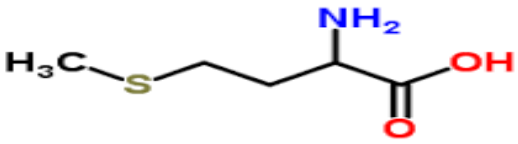

Figure 1.2: Molecular Structure of Methionine.

IUPAC Name : **2-amino-4-(methylsulfanyl)butanoic acid**

Molecular formula : $C_5 H_{11}NO_2 S$

Molecular weight : 149.21g/mol

Pharmacokinetic Data

Bioavailability : 100%

Protein binding : 95%

Metabolism : Hepatic

Excretion : 60-70% Renal, 10-20% Feces

Nicotinamide

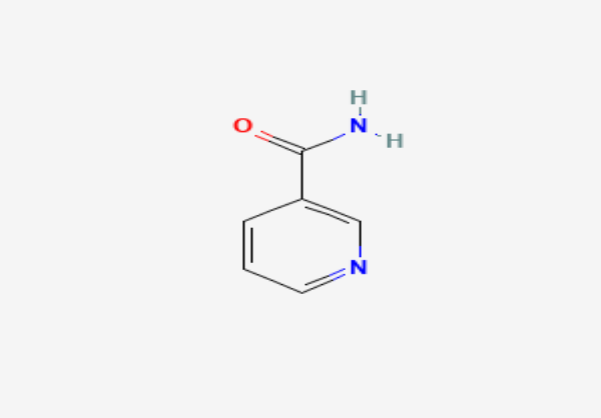

Figure 1.3: Molecular Structure of Nicotinamide.

IUPAC Name : **pyridine-3-carboxamide**

Molecular formula : $C_6H_6N_2O$

Molecular weight : 122.12 g/mol

Pharmacokinetic Data

Bioavailability : 90%

Protein binding : 0-4%

Metabolism : Liver

Excretion : Renal

REVIEW OF LITERATURE:

1. Sharma et al. (2024)[1] developed and validated a robust reverse-phase high-performance liquid chromatography method for the simultaneous estimation of water-soluble vitamins including pyridoxine hydrochloride and nicotinamide in solid dosage forms. Chromatographic separation was achieved on a C18 column using a phosphate buffer–methanol mobile phase with UV detection, and the method was validated as per ICH guidelines.

2. Kumar and Patel (2023)[2] reported a stability-indicating RP-HPLC method for simultaneous determination of pyridoxine hydrochloride and nicotinamide in multivitamin

tablets. Forced degradation studies under acidic, basic, oxidative, thermal, and photolytic conditions demonstrated the specificity of the method.

3. Rao et al. (2023)[3] developed a gradient RP-HPLC method for quantitative estimation of amino acids and B-complex vitamins including DL-methionine and pyridoxine hydrochloride in combined pharmaceutical formulations. Adequate resolution was achieved with acceptable system suitability parameters.

4. Singh et al. (2022)[4] established a validated RP-HPLC method for simultaneous determination of nicotinamide and pyridoxine hydrochloride in nutraceutical products using UV detection. The method showed good linearity, precision, and accuracy.

EXPERIMENTAL

Chemicals and Reagents

Table No.1: List of Chemicals and reagents.

S.NO.	NAME	MANUFACTURER	GRADE
1.	Pyridoxine Hydrochloride, Methionine, Nicotinamide	Atom Pharma (Surat, Gujarat), Anmol Chemicals (mmmmmmmmmm Mu	-
2.	Dummy pellets 16 # 20	Jackson	-
3.	Disodium hydrogen orthophosphate	Merck	GR
4.	Ortho phosphoric acid	Merck	GR
5.	Acetontrile	Merck	HPLC
6.	0.45 µm Nylon filter	Axivia	S0761009

Equipment/Instrument Details

Table No.2: List of Equipment/Instrument details.

S.NO.	INSTRUMENT NAME	MODEL
1.	HPLC system	Agilent 1220 Infinity LC(G4288C)
2.	Analytical balance	Shimadzu
3.	pH Meter	Thermo electron corporation orion 2 star
4.	Sonicator	Ultrasonic cleaner power sonic 420

METHOD DEVELOPMENT

Chromatographic Conditions (Optimized)

Parameter	Condition
Column	C18 column (250 mm × 4.6 mm, 5 µm)
Mobile Phase	0.02 M KH_2PO_4 buffer : Methanol (70:30 v/v)
pH	3.0 (adjusted with OPA)

Flow Rate	1.0 mL/min
Detection Wavelength	260 nm
Injection Volume	20 µL
Column Temperature	30°C
Run Time	10–12 min

Preparation of Solutions

(A) Preparation of Mobile Phase

1. Prepare 0.02 M phosphate buffer.
2. Adjust pH to 3.0 using orthophosphoric acid.
3. Mix buffer and methanol in 70:30 ratio.
4. Filter through 0.45 µm membrane.
5. Sonicate to degas.

(B) Preparation of Standard Stock Solution

1. Accurately weigh:
- Pyridoxine HCl (e.g., 10 mg)
- DL-Methionine (10 mg)
- Nicotinamide (10 mg)
2. Transfer to 100 mL volumetric flask.
3. Add mobile phase.
4. Sonicate for 10 minutes.
5. Make volume up to mark.

Further dilute to obtain working concentrations.

(C) Preparation of Sample Solution

1. Weigh 20 tablets.
2. Calculate average weight.
3. Crush tablets to fine powder.
4. Weigh quantity equivalent to label claim.
5. Transfer to 100 mL volumetric flask.
6. Add mobile phase.
7. Sonicate for 20 minutes.
8. Filter through 0.45 µm filter.
9. Dilute appropriately.

Method Development Strategy

Step 1: Wavelength Selection

- Scan individual drugs in UV (200–400 nm).
- Select common wavelength (260 nm gives good absorbance for all three).

Step 2: Mobile Phase Optimization

- Trials with:
- Water: Methanol
- Buffer: Methanol
- Buffer: Acetonitrile
- Best peak shape obtained with phosphate buffer (pH 3.0) and methanol.

Step 3: pH Optimization

- Tested pH 2.5–4.5.
- Best resolution and symmetry at pH 3.0.

Step 4: Flow Rate Optimization

- 0.8–1.2 mL/min tested.
- 1.0 mL/min gave proper separation and acceptable retention time.

System Suitability Parameters

Before analysis, evaluate:

- Retention time
- Theoretical plates (N > 2000)
- Tailing factor (< 2)
- Resolution (> 2 between peaks)
- %RSD of peak area (< 2%)

Calculation

The content of the drug per average of the capsule was calculated using the formula given the following eqn.

$$\% \text{ of Content} = \frac{\text{Area of Sample} \times \text{Wt. of Std} \times \text{Potency of Std} \times \text{Dilution Factor}}{\text{Peak Area of Std.} \times \text{Wt. of sample} \times 100 \times \text{Avg. Wt.}} \times 100$$

RESULTS AND DISCUSSION

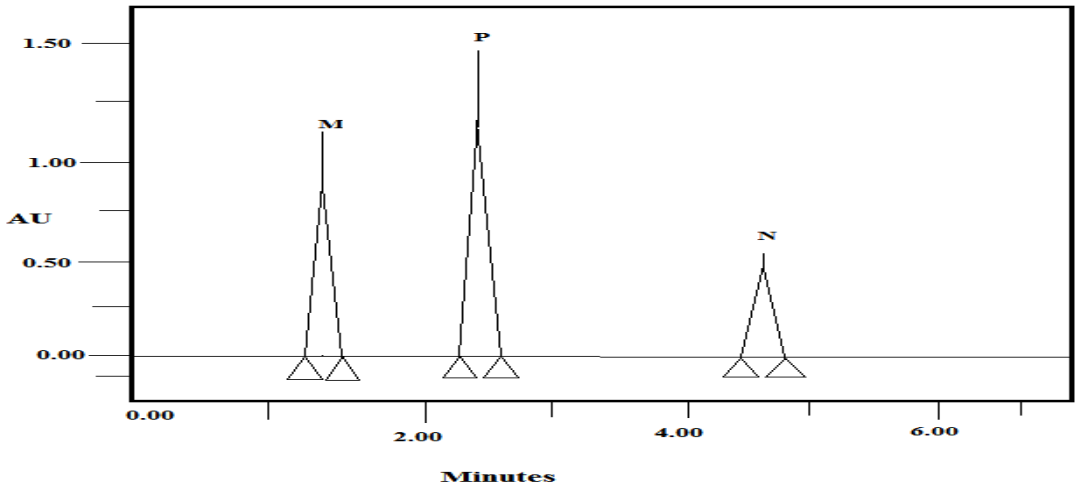

Fig.: Standard chromatogram for Methionine, Pyridoxine hydrochloride and Nicotinamide

S.No.	Parameter	Value
1	Column	Inertsil- ODS C18 (250mm×4.6mm, Particle size 5µm)
2	Mobile Phase	Water (PH 5.2 adjusted with sodium acetate) and methanol in the ratio of 600:400 (v/v)
3	Flow Rate	1.0 ml/min.
4	Diluent	Mobile phase
5	Column Temp.	25 ℃
6	PH	5.2
7	API Concentration	Methionine – 20µg/ml Pyridoxine hydrochloride – 20µg/ml Nicotinamide – 5µg/ml
8	Run Time	6 min.
9	Rentention Time	Methionine – 1.4 min. Pyridoxine hydrochloride – 2.2 min. Nicotinamide – 4.4 min.
10	Volume of Injection	10µL
11	Detection wave Length	247nm

Table 3: Optimized Chromatographic conditions.

S.No.	Concentration (µg/mL)	Peak area	
1	10.00	1877189	Slope =37490 C.C = 0.99 (≈1.0)
2	15.00	2812563	
3	20.00	3747683	
4	25.00	4688354	
5	30.00	5621489	

Table 4: Linearity data of Methionine.

S.No.	Concentration (µg/mL)	Peak area	
1	10.00	3099184	Slope =61968 C.C = 0.99 (≈1.0)
2	15.00	4642641	
3	20.00	6197340	
4	25.00	7744800	
5	30.00	9298119	

Table 5: Linearity data of Pyridoxine Hydrochloride.

S.No.	Concentration (µg/mL)	Peak area	
1	2.5	1793210	Slope =35929 C.C = 0.99 (≈1.0)
2	3.75	2694269	
3	5.00	3593031	
4	6.25	4491038	
5	7.5	5390636	

Table 6: Linearity data of Nicotinamide.

Parameters	Methionine	Pyridoxine hydrochloride	Nicotinamide
Correlation Coefficient	0.99 (≈1.0)	0.99 (≈1.0)	0.99 (≈1.0)
Regression Equation	y = 37490×	y = 61968×	y = 35929×
Theoretical plates	4124	5888	5640
Tailing	1.622	1.180	1.062

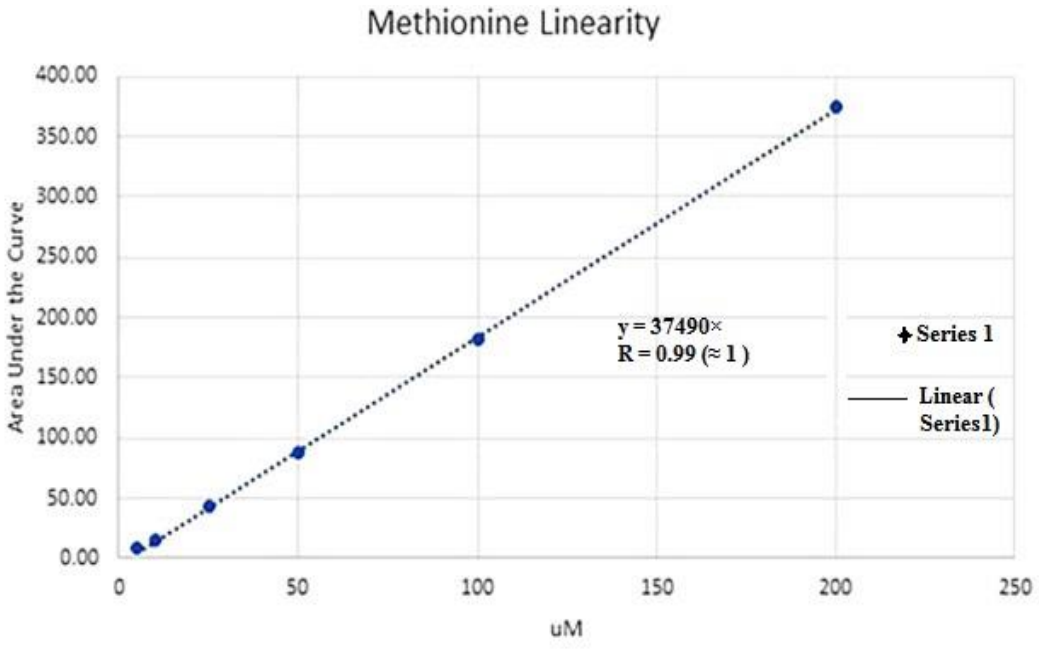

Fig.: Linearity Curve for Methionine.

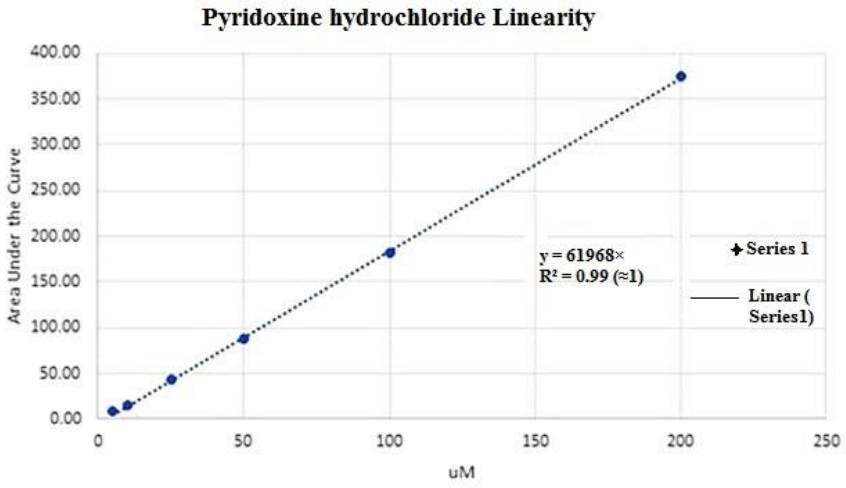

Fig.: Linearity Curve for Pyridoxine Hydrochloride.

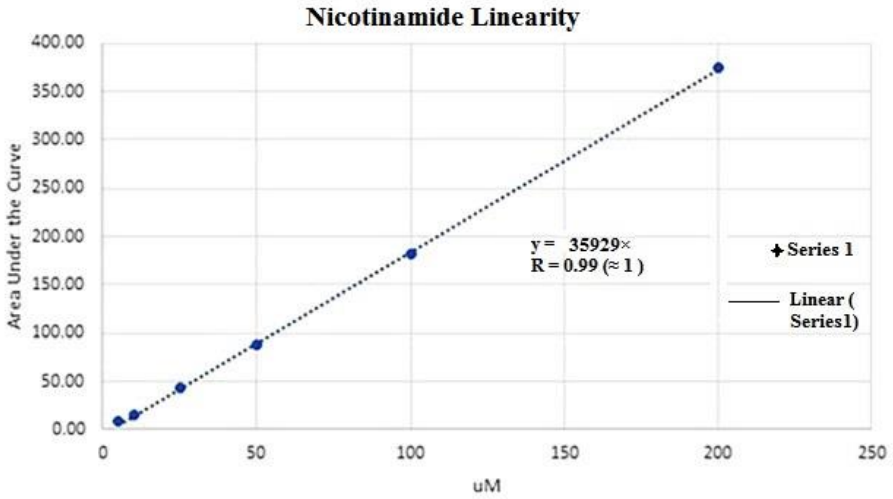

Fig.: Linearity Curve for Nicotinamide.

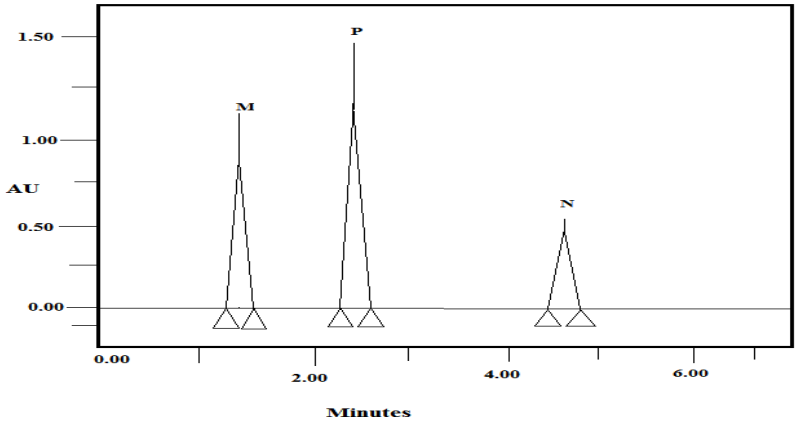

Fig.: Overlay chromatogram of Linearity for Methionine, Pyridoxine Hydrochloride and Nicotinaide

Table 7: Regression characteristics of the linearity plot of Methionine, Pyridoxine hydrochloride and Nicotinamide.

S. No	Sample Weight (mg)	Methionine	Pyridoxine Hydrochloride	Nicotinamide	%Assay (Methionine)	%Assay(Pyridoxine Hydrochloride)	% Assay(Nicotinamide)
1	482.20	3746397	6199675	3595004	99	100	99
2	482.20	3746266	6195760	3592678	99	100	99
3	482.20	3741869	6197389	3590231	99	100	99
4	482.20	3740761	6199444	3591778	99	100	99
5	482.20	3740569	6195273	3599453	99	100	99
6	482.20	3749990	6195000	3593793	99	100	99
Average Assay					99	100	99
STD					0.10	0.03	0.09
% RSD					0.10	0.03	0.09

Table 8: Intra-day precision of Methionine, Pyridoxine hydrochloride and Nicotinamide.

S. No	Sample Weight (mg)	Methionine	Pyridoxine Hydrochloride	Nicotinamide	%Assay (Methionine)	%Assay(Pyridoxine Hydrochloride)	% Assay(Nicotinamide)
1	482.20	3746548	6198328	3595456	99	100	99
2	482.20	3745985	6195284	3594689	99	100	99
3	482.20	3746892	6194983	3594867	99	100	99
4	482.20	3748657	6195749	3598746	99	100	99
5	482.20	3746829	6194862	3598743	99	100	99
6	482.20	3748219	6194168	3598749	99	100	99
Aver					99	100	99

age Assay							
STD					0.03	0.02	0.06
% RSD					0.03	0.02	0.06

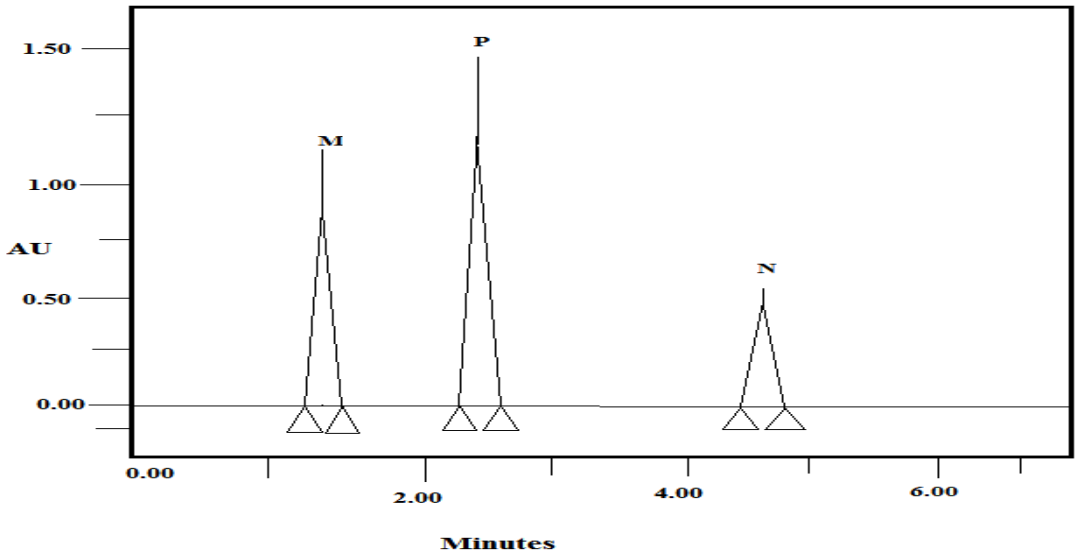

Fig.: Chromatogram of robustness study of Methionine, Pyridoxine Hydrochloride and Nicotinamide

CONCLUSION

Analytical Method Development and Validation for the Determination of Pyridoxine Hydrochloride, DI Methionine and Nicotinamide in Tablet Dosage Form Using Rp HPLC Method was done. First it is discussed about drug of Pyridoxine Hydrochloride, DI Methionine and Nicotinamide, second the materials and methods used in this work, the method development and the methodology of the validation parameters using HPLC, then third the results of system suitability, specificity, linearity, accuracy, precision, repeatability, intermediate precision and robustness.

The application of this method in routine analysis can be justified since easy sample preparation steps are involved and simple reagents, solvents were used experimentally. The method was validated as per ICH guidelines which demonstrated that the procedure is suitable for the intended purpose as it is linear, accurate, precise, rugged, robust, suitable and specific. It shows that the developed HPLC method could be conveniently adopted for the

routine quality control analysis of Pyridoxine Hydrochloride, DI Methionine and Nicotinamide form its pharmaceutical formulation and bulk drug.

REFERENCES

1. Sharma A., Verma S., Gupta R., 2024, Development and validation of RP-HPLC method for simultaneous estimation of water-soluble vitamins in pharmaceutical dosage forms, Journal of Pharmaceutical Analysis, 14, 112–120.
2. Kumar S., Patel R., 2023, Stability-indicating RP-HPLC method for simultaneous determination of pyridoxine hydrochloride and nicotinamide in tablets, International Journal of Pharmaceutical Sciences and Research, 14, 2560–2568.
3. Rao P., Naik S., Reddy M., 2023, Gradient RP-HPLC method for estimation of amino acids and B-complex vitamins in combined dosage forms, Asian Journal of Pharmaceutical Analysis, 13, 45–52.
4. Singh V., Meena K., Sharma D., 2022, Validated RP-HPLC method for simultaneous estimation of nicotinamide and pyridoxine hydrochloride, Journal of Applied Pharmaceutical Science, 12, 089–095.
5. Ahmed M., Khan A., Hussain T., 2022, RP-HPLC method development for DL-methionine with B-complex vitamins in tablet dosage form, International Journal of Pharmaceutical Chemistry, 9, 33–40.
6. Patel K., Shah N., Desai J., 2021, Simultaneous estimation of DL-methionine, pyridoxine hydrochloride and nicotinamide by RP-HPLC, Asian Journal of Research in Pharmaceutical Sciences, 11, 101–107.
7. Saini P., Mandal S., Agarwal A., 2021, Development and validation of RP-HPLC method for simultaneous determination of DL-methionine, pyridoxine hydrochloride and nicotinamide in tablets, American Journal of PharmTech Research, 11, 245–255.
8. Gupta N., Jain A., Kumar V., 2020, Stability-indicating RP-HPLC method for water-soluble vitamins in pharmaceutical formulations, Journal of Drug Delivery and Therapeutics, 10, 120–126.
9. Zohora F., Rahman M., Islam S., 2020, Development and validation of RP-HPLC method for simultaneous estimation of B-complex vitamins in multivitamin preparations, European Journal of Pharmaceutical and Medical Research, 7, 178–185.
10. Mehta D., Joshi H., Patel P., 2019, RP-HPLC determination of methionine in pharmaceutical and nutraceutical products, International Journal of Pharmaceutical Analysis, 8, 67–73.

11. Chaudhary A., Yadav R., Singh P., 2019, Simultaneous estimation of nicotinamide and pyridoxine hydrochloride by RP-HPLC, World Journal of Pharmacy and Pharmaceutical Sciences, 8, 945–955.
12. Reddy K., Prasad V., Rao J., 2018, Stability-indicating RP-HPLC method for estimation of pyridoxine hydrochloride in combined dosage forms, Journal of Chromatographic Science, 56, 412–418.
13. Kaur H., Kaur M., Singh G., 2018, RP-HPLC method for simultaneous determination of methionine and B-complex vitamins, International Journal of Pharmaceutical Research, 10, 210–216.
14. Vidović S., Stojanović B., Veljković J., 2017, Stability-indicating HPLC method for determination of water-soluble vitamins in pharmaceuticals, Acta Chromatographica, 29, 123–134.
15. Gliszczyńska-Świgło A., 2016, Simultaneous determination of water-soluble vitamins by HPLC-PDA method, Food Chemistry, 172, 403–410.
16. Jeyalakshmi R., Venkatesh P., Kumar M., 2015, Development and validation of RP-HPLC method for simultaneous estimation of vitamins B1, B3 and B6, International Journal of Pharmacy and Pharmaceutical Sciences, 7, 210–215.
17. Perveen S., Yasmin A., Khan A., 2014, Simultaneous determination of water-soluble vitamins in pharmaceutical formulations by RP-HPLC, Pakistan Journal of Pharmaceutical Sciences, 27, 175–181.
18. Markopoulou C., Kagkadis K., Koundourellis J., 2013, Application of RP-HPLC for the determination of B-complex vitamins, Journal of Pharmaceutical and Biomedical Analysis, 81, 187–196.