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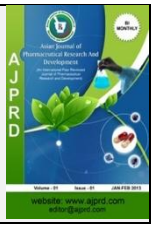

Open Access Research Article

Development and Validation of RP-HPLC Method for the Simultaneous Estimation of Methionine, Pyridoxine Hydrochloride and Nicotinamide in Combined Dosage Form

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A B S T R A C T

Analytical chemistry is one of the important branches of science which deals with the study of separation, identification and quantification of various natural and chemical compounds. Quantitative analysis is having supremacy from the point of pharmaceutical chemistry. Quantification methods include chromatographic techniques, spectrophotometery, fluorimetery etc. One of the accurate method for quantitative analysis is High Performance Liquid Chromatography (HPLC). In the present manuscript, a reverse phase – HPLC method was developed for the simultaneous determination of Methionine, Pyridoxine hydrochloride and Nicotinamide in combined dosage form and validation of the developed method. The development of method includes selection of mobile phase, chromatographic method and wavelength whereas validation involves the parameters like linearity, accuracy, precision, Limit of detection (LOD), Limit of Quantification (LOQ), robustness, system suitability of the developed method. The result showed that the developed method is best suited to the simultaneous determination of Methionine, Pyridoxine hydrochloride and Nicotinamide and validated as per standards.

Keywords: Validation, HPLC, accuracy, precision

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INTRODUCTION

Analytical chemistry is the study of the separation, identification, and quantification of the chemical components of natural and artificial materials [1]. Qualitative analysis gives an indication of the identity of the chemical species and amount of one or more of these components. Analytical chemists perform qualitative and quantitative analysis; use the science of sampling, defining, isolating, concentrating, and preserving samples; set error limits; validate and verify results through calibration and standardization. In analysis various techniques are being used for evaluation purpose. Looking at the current scenario hybrid or hyphenated analysis techniques are employed for better interpretation. A hyphenated separation technique refers to a combination of two (or more) techniques to detect and separate chemicals from solutions. For example, gas chromatography-mass spectrometry(GC-MS), gas chromatography infrared spectroscopy(GC-IR), liquid chromatography-mass spectrometry (LC-MS), Liquid chromagraphy-infrared spectroscopy and capillary electrophoresis-mass spectrometry etc. [5] The present study, therefore, aims at developing specific, precise, accurate optimized and validated HPLC. High-performance liquid chromatography (or high-pressure liquid chromatography, HPLC) is a chromatographic technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture. There are two variants in use in HPLC depending on the relative polarity of the solvent and the stationary phase. Normal phase HPLC, Reversed phase HPLC [20, 21].

EXPERIMENTAL WORK

The present study, therefore, aims at developing specific, precise, accurate optimized and validated HPLC methods for the Nicotinamide Pyridoxine HCl and Methionine in combined dosage forms. It is proposed therefore to develop and optimize the chromatographic conditions like selection of initial separation conditions, nature of the stationary phase, nature of mobile phase, sensitivity, selection of solvents and reagents and selection of internal standard. [11]

After the method development, the drugs in the formulations are proposed to be estimated and the method developed is validated using the various validation performance parameters such as accuracy, precision (repeatability and reproducibility), linearity and range, limit of detection (LOD)/limit of quantitation (LOQ), robustness/ruggedness, stability and system suitability.

Instrument used

The author had developed a liquid chromatographic method in bulk samples and pharmaceutical formulations. In this study PEAK 7000 isocratic HPLC with rheodine manual sample injector with switch (77251) was employed and the column used was thermohypersil BDS C18 (250 mmx4.6 mm, particle size 5 µm) column, Waters 2695 alliance with binary HPLC pump and a Waters 2998 PDA detector. Waters Empower 2 software was used for monitoring chromatographic analysis and data acquisition. Spectra lab DGA 20 A3 ultrasonic bath sonicator was used for degassing the mobile phase. Electronic balance ELB 300 was used for weighing the materials. The syringe used for injecting was 20 µL Hamilton syringe. DIGISUN pH meter was used for all pH measurements. [6-8]

Drugs

The working standards of Methionine, Pyridoxine hydrochloride and Nicotinamide were provided as gift samples from Dr. Reddy's Laboratories Ltd, Hyderabad. Marketed formulation of combination was purchased from local market. [7]

Chemicals and reagents

Methanol (HPLC grade), sodium acetate solution (IR grade), water (Triple distilled water was prepared by using Borosil Glass Distillation Unit)

Mobile phase

The mobile phase composition used for elution was 0.01M sodium acetate solution and methanol in the ratio of 600:400 (v/v). It was prepared by diluting 400 mL methanol and 600 mL water (pH 5.2 adjusted with sodium acetate) in one litre flask. It was filtered through 0.45 µ nylon membrane filter before use. This mixture was also used as diluents for preparing working standard solutions of the drug. [9]

Stock and working standard solutions

Pure standards of Methionine, Pyridoxine hydrochloride and Nicotinamide were used as external standards in the analysis. A standard stock solution of 1 mg/mL of Methionone, Pyridoxine hydrochloride and Nicotinamide were prepared separately used methanol as solvent. In order to get the required ratio (4:4:1) of the drugs Methionine, Pyridoxine hydrochloride and Nicotinamide, appropriate quantities of respective solutions of each drug were mixed and diluted with the mobile phase. The flask containing standard solution was sonicated for 10 minutes to degas it. The standard solution was then filtered with 0.45 µm membrane filter paper. A series of different dilutions (50-100 µg/mL) were prepared using above stock solution with selected mobile phase and analyzed using the same chromatographic conditions as those of the target compounds and a calibration curve was generated. [12-14]

Sample preparation

Accurately weighed quantity of sample powder equivalent to 10 mg of Methionine, 10 mg of Pyridoxine hydrochloride and 2.5 mg of Nicotinamide was transferred into 100 mL of volumetric flask added 50mL of water and sonicated for 30 min and make up the volume with mobile phase and filtered through the 0.45 µm membrane filter paper. 5 mL of the above solution is taken into 25 mL volumetric flask make up the volume with mobile phase. An aliquot of this solution was injected into HPLC system. [15]

RESULTS

The following optimized chromatographic conditions mentioned in Table 1 were followed for the determination of Methionine, Pyridoxine hydrochloride and Nicotinamide in bulk samples and pharmaceutical formulations.

Table 1: Optimized chromatographic conditions

S.No	Parameters	Values
1	Column	Inertsil- ODS C18 (250 mmx4.6 mm, particle size 5 µm)
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 temperature	25°C
6	pH	5.2
7	API Concentration	Methionine - 20 µg/mL Pyridoxine hydrochloride- 20 µg/mL Nicotinamide - 5 µg/mL
8	Run time	6 min
9	Retention time	Methionine -1.4 min,Pyridoxine hydrochloride-2.2 min, Nicotinamide -4.4 min.
10	Volume of injection	10 µL
11	Detection wave length	247 nm

Validation of the Developed Method

The developed method was validated in terms of different parameters like linearity, specificity, precision, accuracy, LOD & LOQ in compliance with ICH [19] guide lines.

Linearity

Linearity of the peak areas were determined by taking six replicate measurements. The linearity data was reported in Table 2, Table 3 and Table 4. The statistical parameters of the linearity plots were show in Table 5. The results showed that an excellent correlation exists between areas and concentration of drugs within the concentration range indicated above. [21.]

Table 2: Linearity data of Methionine

S.No	Concentration (µg/mL)	Peak area	
1	10	1877189	Slope = 37490 C.C = 0.99 (~1.0)
2	15.00	2812563	
3	20.00	3747683	
4	25	4688354	
5	30	5621489	

Table 3: Linearity data of Pyridoxine hydrochloride

S.No	Concentration (µg/mL)	Peak area	
1	10	3099184	Slope = 61968 C.C = 0.99 (~1.0)
2	15	4642641	
3	20	6197340	
4	25	7744800	
5	30.00	9298119	

Table 4: Linearity data of Nicotinamide

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 5: Regression characteristics of the Linearity plot of Methionine, Pyridoxine hydrochloride and Nicotinamide

Parameters	Methionine	Pyridoxine hydrochloride	Folic acid
Correlation Coefficient	0.99 (~1.0)	0.99 (~1.0)	0.99 (~1.0)
Regression Equation	y = 37490 x	y = 61968x	y = 35929 x
Theoretical plates	4124	5888	5640
Tailing	1.622	1.180	1.062

Table 6: 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	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 7: Inter – 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
Average Assay:					99	100	99
STD					0.03	0.02	0.06
%RSD					0.03	0.02	0.06

Table 8: Accuracy data of Methionine

Spiked Level	Sample Weight (mg)	Sample Area	µg/mL added	µg/mL found	% recovery	Mean
50%	241.50	1875005	9.910	9.92	100.10 (~100)	100.04 (~100)
50%	241.50	1875013	9.910	9.92	100.10 (~100)	
50%	241.50	1879474	9.910	9.94	100.30 (~100)	
50%	241.50	1871012	9.910	9.90	99.89 (~100)	
50%	241.50	1870158	9.910	9.89	99.79 (~100)	
50%	241.50	1876423	9.910	9.92	100.10 (~100)	
100%	483.00	3747599	19.820	19.82	100.00 (~100)	99.99 (~100)
100%	483.00	3745976	19.820	19.81	99.94 (~100)	
100%	483.00	3749990	19.820	19.83	100.05 (~100)	

Table 9: Accuracy data of Pyridoxine hydrochloride

Spiked Level	Sample Weight (mg)	Sample Area	µg/mL added	µg/mL found	% recovery	Mean
50%	241.50	3099476	9.970	9.98	100.10 (~100)	100.03 (~100)
50%	241.50	3097631	9.970	9.97	100.00(~100)	
50%	241.50	3097853	9.970	9.97	100.00(~100)	
50%	241.50	3099674	9.970	9.98	100.10 (~100)	
50%	241.50	3099739	9.970	9.98	100.10 (~100)	
50%	241.50	3092668	9.970	9.96	99.89 (~100)	
100%	483.00	6199374.00	19.940	19.96	100.10 (~100)	100.03 (~100)
100%	483.00	6193618.00	19.940	19.94	100.00(~100)	
100%	483.00	6195000.00	19.940	19.94	100.00(~100)	

Table 10: Accuracy data of Nicotinamide

Spiked Level	Sample Weight (mg)	Sample Area	µg/mL added	µg/mL found	% recovery	Mean
50%	241.50	1796227	2.478	2.47	99.67 (~100)	99.80 (~100)
50%	241.50	1797935	2.478	2.48	100.08 (~100)	
50%	241.50	1794166	2.478	2.47	99.67 (~100)	
50%	241.50	1798889	2.478	2.48	100.08 (~100)	
50%	241.50	1796892	2.478	2.47	99.67 (~100)	
50%	241.50	1793796	2.478	2.47	99.67 (~100)	
100%	483.00	3595593	4.955	4.95	99.89 (~100)	99.89 (~100)
100%	483.00	3590965	4.955	4.95	99.89 (~100)	
100%	483.00	3593793	4.955	4.95	99.89 (~100)	

Limit of detection and Limit of Quantification (LOD&LOQ) study

LOD is the smallest concentration of the analyte which gives a measurable response. It is calculated by taking the concentration of the peak of interest divided by three times the signal to noise ratio (s/n). LOQ is the smallest concentration of the analyte, which gives response that can be absolutely quantified. It is determined by analyzing samples containing known quantities of the analyte and determining the lowest level at which acceptable degrees of accuracy and precision are attainable.

The limit of detection and limit of quantification were evaluated by serial dilutions of Methionine, Pyridoxine hydrochloride and Nicotinamide stock solutions by the proposed method in order to obtain signal to noise ratio of 3:1 for LOD and 10:1 for LOQ. The LOD value for Methionine, Pyridoxine hydrochloride and Nicotinamide were found to be 0.050, 0.0370 and 0.033 respectively and the LOQ value 0.167, 0.1233 and 0.109 respectively.

Table 11: LOD and LOQ of Methionine

LOD	0.050
LOQ	0.167

Table 12: LOD and LOQ of Pyridoxine hydrochloride

LOD	0.0370
LOQ	0.1233

Table 13: LOD and LOQ of Nicotinamide

LOD	0.033
LOQ	0.109

Table 14: Ruggedness of Methionine

Injection number	**Peak area**
1	3742362
2	3740123
3	3739472
4	3720912
5	3701634
6	3692345
7	3681204
Mean	3716864.5714
SD	23370.9432
% RSD	0.62878

Table 15: Ruggedness of Pyridoxine hydrochloride

Injection number	Peak area
1	6197213
2	6196234
3	6183723
4	6173210
5	6163846
6	6153012
7	6140371
Mean	6172515.571
SD	19955.0915
% RSD	0.32328

Table 16: Ruggedness of Nicotinamide

Injection number	Peak area
1	3599456
2	3585012
3	3580835
4	3575031
5	3570237
6	3565023
7	3560284
Mean	3576554
SD	12272.3103
% RSD	0.343132

Estimation of the drug in formulation:

Accurately weighed Quantity of sample powder equivalent to 10 mg of Methionine, 10 mg of pyridoxine hydrochloride and 2.5 mg of Nicotinamide was transferred into 100 mL of volumetric flask added 50 mL of water and sonicated for 30 mins and make up the volume with mobile phase and filtered through the 0.45 µm membrane filter paper. From the above solution, take 5mL into 25 mL volumetric flask make up the volume with mobile phase. An aliquot of this solution was injected into HPLC system. Peak areas of sample were measured and compared against the peak areas of the standard solution. The proposed method was able to estimate Methionine, Pyridoxine hydrochloride and Nicotinamide in the formulation with accuracy of 99.9% (~100%) for Methionine, 98.95% (~100%) for Pyridoxine hydrochloride and 99.8% (~100%) for Nicotinamide. The results were tabulated and the percentage assay was reported in Table 20.

Table 20: Estimation of Methionine, Pyridoxine hydrochloride and Nicotinamide from its formulation

Formulation	Dosage	Sample conc. µg/mL	Sample Area	Amount Found µg/mL	% Assay
Becilan	Methionine	20	3747649.1	19.98	99.9
	Pyridoxine hydrochloride	20	6197298.5	19.79	98.95
	Nicotinamide	5	3593276.4	4.99	99.8

DISCUSSION

Method development

Several mobile phase compositions were tried to get good optimum resolutions of Methionine, Pyridoxine hydrochloride and Nicotinamide peaks. The mobile phase containing water (pH 5.2 adjusted with sodium acetate) and methanol in the ratio of 600: 400 (v/v) was selected because it gave sharp peaks with good resolution, minimum tailing and satisfactory retention time. The drugs having appreciable absorbance at 247 nm and therefore 247 nm was selected as the detection wavelength. The working standard solutions of Methionine, Pyridoxine hydrochloride and Nicotinamide were injected separately. The retention time of Methionine, Pyridoxine hydrochloride and Nicotinamide was found to be 1.4 min, 2.2 min and 4.4 min respectively when injected as individual compounds.

Validation of the method :The validation of the method was done by various methods in terms of linearity, precision, accuracy, robustness, LOD/LOQ, robustness and ruggedness/system suitability as per ICH guidelines. [16-19]

To evaluate the linearity of method, six replicate measurements were done. The linearity range of Methionine, Pyridoxine hydrochloride and Nicotinamide were found 10-30 µg/mL, 10-30 µg/mL and 2.5-7.5 µg/mL respectively. The obtained data demonstrates that method has sufficient sensitivity to the concentrations of analytes.

The precision of the method was determined by performing six independent assays of the test samples and inter-day precision was checked by doing same procedure on different days by another person under the same experimental conditions. The %RSD for intra-day precision for Methionine, Pyridoxine hydrochloride, Nicotinamide was found to be 0.10, 0.03, 0.09 respectively. The %RSD for inter-day precision for Methionine, Pyridoxine hydrochloride, Nicotinamide was obtained 0.03, 0.02, 0.06 respectively. Hence the method was showing high degree of precision. [9]

The accuracy of the method was evaluated by doing recovery studies. The recovery experiments were performed at three concentrations levels i.e. 50, 100 and 150%. Each level was repeated for six times. The recovery for Methionine, Pyridoxine hydrochloride, Nicotinamide were found to be 99.9%, 98.95% and 99.8% respectively. So the method is adequately accurate.

The LOD/LOQ were determined by serial dilutions of Methionine, Pyridoxine hydrochloride, Nicotinamide stock solutions by the proposed method. [10=[-] The LOD values for Methionine, Pyridoxine hydrochloride, Nicotinamide were found to be 0.050, 0.0370 and 0.033 respectively and the LOQ values for Methionine, Pyridoxine hydrochloride, Nicotinamide were 0.167, 0.123, 0.109 respectively.The robustness of the method was checked by doing certain experiments using changing conditions like flow rate and temperature. No significant effect was seen on chromatographic resolution and hence the developed method was found to be robust.

The system suitability was checked by injecting seven replicates of working standard solution at six min interval. The % RSD for Methionine, Pyridoxine hydrochloride, Nicotinamide were found to be 0.628, 0.323, 0.343 respectively. So the system was found to be suitable for the determination of Methionine, Pyridoxine hydrochloride, Nicotinamide.

CONCLUSION

The statistical evaluation of the proposed method revealed its good linearity, reproducibility and its validation for different parameters made us to conclude that the current RP-HPLC method can successfully be used for reliable determination of Methionine, Pyridoxine hydrochloride and Nicotinamide in pharmaceutical dosage from and also in bulk drug. The developed method was found specific to the drug and for dosage from because no interfering material peaks near the drug peak were observed in the chromatograms obtained in the study runtime.

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