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METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF NICOTINIC ACID, FOLIC ACID AND CYANOCOBALAMIN USING RP-HPLC

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ABSTRACT

A simple Reverse phase liquid chromatographic method has been developed and subsequently validated for simultaneous determination of nicotinic acid, folic acid and cyanocobalamin in combination. The separation was carried out using a mobile phase consisting of sodium acetate buffer of pH 2.5 and Acetonitrile in the ratio of 90 : 10. The column used was phenomenex Luna 5 μ c18 (2) 250x4.6 mm, 100 Å with flow rate of 1.5 ml/min, and the detection was done at 278 nm. The retention times of nicotinic acid, folic acid and cyanocobalamin were found to be 3.79, 6.01 and 14.54 min respectively. The method was validated for linearity, accuracy, precision, limit of detection, limit of quantification and ruggedness. The limit of detection for nicotinic acid, folic acid and cyanocobalamin was found to be 100 ng/ml, 1 μ g/ml and 10 μ g/ml and the limit of Quantification was found to be 1 μ g/ml, 10 μ g/ml and 100 μ g/ml respectively. Linearity for nicotinic acid, folic acid and cyanocobalamin has been observed in the range of 1-5 μ g/ml, 10-50 μ g/ml and 100-500 μ g/ml and the respective R^2 values are observed at 0.996, 0.997 and 0.99 respectively. The relative standard deviation for intra and interday precision were <5%. Hence the proposed RP-HPLC method was sensitive, rapid, cost-effective and accurate for the simultaneous determination of nicotinic acid, folic acid and cyanocobalamin in both bulk materials and pharmaceutical dosage forms.

Key words: Nicotinic acid, Folic acid, Cyanocobalamin, Validation.

INTRODUCTION

Nicotinic acid [NA] (pyridine-3-carboxylic acid) is a B group vitamin. It is one of the oldest known agents for the treatment of dyslipidaemia. It favorably modifies the major lipid and lipoprotein classes that are recognized as independent risk factors for atherosclerosis and coronary heart disease¹. It can be used as a therapeutic option for cardiovascular risk reduction in patients with abnormal glucose metabolism and atherogenic dyslipidaemia². Chronic administration of Nicotinic acid reduces whole-body insulin sensitivity via elevation of circulating fatty acids (to high physiologic levels) and accumulation of skeletal muscle lipid³. Nicotinic acid and its derivatives can be used as peripheral vasodilators⁴. Nicotinic acid can resist insulin which was studied by measuring euglycemic-hyperinsulinemic clamp which is induced by 2 weeks administration^{5,6}. Folic acid [FC] (2S)-2-[(4-[(2-amino-4-hydroxypteridin-6-yl) methyl] amino) phenyl) formamido] pentanedioic acid is a water soluble vitamin. Megaloblastic anemia is the result of Folic acid deficiency and one of the chemical signs of acute folate deficiency includes a red, painful tongue. FC is associated in prevention and treatment of depression⁷. Cyanocobalamin α -(5, 6-dimethylbenzimidazolyl) cobamidecyanide is very hygroscopic in the anhydrous form, and sparingly soluble in water (1:80), cyanocobalamin is the commercial form of vitamin B12 and specifications are found in the codex for use as food, and in the USP for pharmaceutical use⁸. Vitamin B12 is closely related, metabolically to Folic acid and takes part in methyl group transfer from N5-methyltetrahydro- Folic acid to homo-cysteine⁹. Folic acid has been shown to reverse endothelial dysfunction induced by inhibition of Tetrahydrobiopterin

biosynthesis, β glycine supplement on uterine metabolism of prostaglandin and endometrial granulocyte-macrophage colony-stimulating factor expression during early pregnancy in pigs^{10,11}. Some Folic acid degradation products have been identified after E-beam irradiation¹². There are various methods for determining Folic acid in food which include biological, microbiological, chemical, chromatographic and radiometric assays, Folic acid can also be determined by using ion-pairing and by coupling reactions with iminodibenzyl or 3-aminophenol or sodium molybdate-pyrocatechol or by using folate binding protein and Folic acid can be preferably determined in formulations, milk and in plasma by using UHPLC (Ultra High Pressure Liquid Chromatography) and LC/MS/MS¹³⁻²⁰. Nicotinic acid can be purified by affinity chromatography using a regioselectively modified and reversibly immobilized α -toxin from *Naja nigricollis*²¹. Removal of cyanocobalamin from aqueous solution using mesoporous activated carbon was determined²². In nutritional supplements Nicotinic acid and Folic acid were determined by using RP-HPLC with UV detection^{23,24}. Water-soluble vitamins can be determined in Nutraceutical Products and in Functional Waters by HPLC with UV-PDA Detection²⁵.

EXPERIMENTAL

Reagents and Chemicals

Vitamins (Nicotinic acid, Folic acid, Cyanocobalamin) were obtained from Strides Arcolab, Bangalore. Acetonitrile and HPLC water were obtained from Merck-Mumbai. Sodium Acetate and Glacial Acetic Acid were obtained from Universal Lab Pvt Ltd-Mumbai.

Instrumentation and Chromatographic Conditions

The HPLC system used was Shimadzu-UFLC of model SPD-20A, consisting of pump LC-20AD for delivering mobile phase to the analytical column. A Rheodyne injector of model 7725i and phenomenex Luna 5 μ c18 (2) 250x4.6 mm, 100 Å was used. The mobile phase consisted of Buffer and Acetonitrile in the ratio 90:10, the buffer used is sodium acetate buffer of pH-2.5, the flow rate was found to be 1.5 ml/min and the detection was done at 262 nm. The retention times of nicotinic acid, folic acid and cyanocobalamin were found to be 3.79, 6.01 and 14.54 minutes respectively.

Preparation of stock solution

1mg/ml solutions of nicotinic acid, folic acid and cyanocobalamin were prepared by taking 10 gm of nicotinic acid, folic acid and cyanocobalamin in 10 ml volumetric flasks individually and made up to 10 ml with 0.1N NaOH. From 1 mg/ml solution of nicotinic acid 1 ml is taken and made up to 10 ml with 0.1N NaOH to make it to 100 μ g/ml which is used as stock solution.

Preparation of calibration standard

From stock solution of nicotinic acid, folic acid 0.1, 0.2, 0.3, 0.4, 0.5 ml were taken into individual volumetric flasks and made upto 10 ml with 0.1N NaOH. From stock solution of Cyanocobalamin 1, 2, 3, 4, 5 ml were taken into individual volumetric flasks and made upto 10 ml with 0.1N NaOH.

Method Validation

Validation of an analytical method is the process by which it is established by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical application. Validation is required for any new or amended method to ensure that it is capable of giving reproducible and reliable results, when used by different operators employing the same equipment in the same or different laboratories. The type of validation programme required depends entirely on the particular method and its proposed applications.

Typical analytical parameters used in method validation include:

- Sensitivity
- Limit of detection
- Limit of quantification
- Linearity and Range
- Precision
- Accuracy
- Stability
- Ruggedness

Sensitivity

Limit of Detection

This is the lowest concentration in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. The limit of detection is important for impurity tests and the assays of dosages containing low drug levels and placebos. The limit of detection is generally quoted as the concentration yielding a signal-to-noise ratio of 3:1. The signal-to-noise ratio is determined by:

$$s = H/h$$

Where H = height of the peak corresponding to the component h = absolute value of the largest noise

fluctuation from the baseline of the chromatogram of a blank solution.

Limit of Quantification

This is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. It is quoted as the concentration yielding a signal-to-noise ratio of 10: 1 and is confirmed by analyzing a number of samples near this value.

Linearity and Range

The range of the method is the interval between the upper and lower levels of an analyte that have been determined with acceptable precision, accuracy and linearity. It is determined on either a linear or nonlinear response curve and is normally expressed in the same units as the test results.

This is the method's ability to obtain results which are either directly, or after mathematical transformation proportional to the concentration of the analyte within a given range. Linearity is determined by calculating the regression line using a mathematical treatment of the results (i.e., least mean squares) vs analyte concentration.

Precision

The precision of an analytical method is the degree of agreement among individual test results obtained when the method is applied to multiple sampling of a homogenous sample. Precision is a measure of the reproducibility of the whole analytical method (including sampling, sample preparation and analysis) under normal operating circumstances. Precision is determined by using the method to assay a sample for a sufficient number of times to obtain statistically valid results (i.e., between 6 - 10). The precision is then expressed as the relative standard deviation:

$$\%RSD = \frac{STD \text{ dev} \times 100\%}{\text{Mean}}$$

Accuracy

Accuracy is a measure of the closeness of test results obtained by a method to the true value. Accuracy is expressed in terms of percentage recovery. It was done by spiking the sample with known amount of standard at three levels of sample concentration.

Stability

In order to demonstrate the stability of nicotinic acid, folic acid and cyanocobalamin solutions during analysis, the solutions were analyzed over a period of 8 hours at room temperature.

Ruggedness

Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions i.e., different analysts, laboratories, instruments, reagents, assay temperatures, small variations in mobile phase, different days etc., (i.e., from laboratory to laboratory, from analyst to analyst).

RESULTS AND DISCUSSION

The limit of detection for nicotinic acid, folic acid and cyanocobalamin was found to be 100 ng/ml, 1 μ g/ml and 10 μ g/ml respectively.

The limit of Quantification for nicotinic acid, folic acid and cyanocobalamin was found to be 1 μ g/ml, 10 μ g/ml and 100 μ g/ml respectively.

Table 1: Intraday precision

	Nicotinic acid			Folic acid			Cyanacobalamin		
	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
	1µg/ml	3µg/ml	5µg/ml	10µg/ml	30µg/ml	50µg/ml	100µg/ml	300µg/ml	500µg/ml
1 st	0.96	3.04	5.05	9.90	30.00	51.20	99.00	298.00	497.00
2 nd	0.98	2.96	5.19	9.90	30.00	51.50	97.00	301.00	499.00
3 rd	1.05	2.99	4.74	10.10	29.00	47.20	99.00	299.00	504.00
AVG	0.99	2.99	4.99	9.96	29.60	49.96	98.33	299.30	500.00
SD	0.03	0.03	0.18	0.09	0.47	1.96	0.94	1.24	2.94
%RSD	3.98	1.14	3.76	0.94	1.58	3.92	0.95	0.41	0.58
%Nominal	99.90	99.90	99.80	99.60	98.86	99.92	98.30	99.76	100.00

Table 2: Interday precision

	Nicotinic acid			Folic acid			Cyanacobalamin		
	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
	1µg/ml	3µg/ml	5µg/ml	10µg/ml	30µg/ml	50µg/ml	100µg/ml	300µg/ml	500µg/ml
1 st week	0.99	2.99	4.99	9.96	29.66	49.96	98.00	294.00	494.00
2 nd week	0.99	2.99	4.99	9.99	29.96	49.96	97.00	308.00	498.00
3 rd week	0.99	2.99	4.99	9.99	29.99	49.99	96.00	296.00	500.00
AVG	0.99	2.99	4.99	9.98	29.87	49.97	97.00	299.30	497.30
SD	0.002	0.001	0.003	0.01	0.14	0.01	0.81	6.18	2.49
%RSD	0.24	0.04	0.07	0.14	0.49	0.02	0.84	2.06	0.50
%Nominal	99.60	99.90	99.90	99.80	99.56	99.94	97.00	99.76	99.46

Table 3: Recovery studies

LEVELS	Nicotinic acid			Folic acid			Cyanacobalamin		
	I 1µg/ml	II 3µg/ml	III 5µg/ml	I 10µg/ml	II 30µg/ml	III 50µg/ml	I 100µg/ml	II 300µg/ml	III 500µg/ml
Conc. of drug added	10µg/ml			10µg/ml			10µg/ml		
Amount of drug recovered	11.05	13.05	15.10	11.08	13.05	15.01	11.02	12.99	15.06
%Recovery	0.11	0.13	0.15	0.11	0.13	0.15	0.11	0.12	0.15
% CV	1.07	0.63	0.61	1.27	1.00	0.73	0.91	0.30	0.98
N	6			6			6		

Table 4: Stability studies

	Nicotinic acid			Folic acid			Cyanacobalamin		
	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
	1µg/ml	3µg/ml	5µg/ml	10µg/ml	30µg/ml	50µg/ml	100µg/ml	300µg/ml	500µg/ml
1 st week	0.99	3.00	4.87	9.90	30.60	50.00	98.00	299.00	498.00
2 nd week	0.97	2.94	5.00	9.00	29.00	49.60	100.00	300.00	495.00
3 rd week	1.00	2.98	4.99	10.00	29.80	50.10	99.00	297.00	500.00
AVG	0.99	2.97	4.95	9.63	29.80	49.90	99.00	298.60	497.60
SD	0.01	0.02	0.05	0.44	0.65	0.21	0.81	1.24	2.05
%RSD	1.11	0.83	1.19	4.66	2.19	0.43	0.82	0.41	0.41
%Nominal	99.10	99.10	99.06	96.30	99.30	99.80	99.00	99.53	99.52

LQC: Low Quality Control, MQC: Medium Quality Control, HQC: High Quality Control

Figure 1: Linearity of nicotinic acid

Figure 2: Linearity of folic acid

Figure 3: Linearity of cyanocobalamin

Figure 4: Typical HPLC chromatogram of nicotinic acid, folic acid and cyanocobalamin mixture.

The linearity of nicotinic acid, folic acid and cyanocobalamin was performed in the range of 1-5 µg/ml, 10-50 µg/ml and 100-500 µg/ml respectively. The R^2 values for the respective compounds were 0.996, 0.997 and 0.99 respectively and the linearity of nicotinic acid, folic acid and cyanocobalamin were shown in figures [1-3].

The intraday and inter-day variations of the method were determined using three replicate injections of different concentrations, analyzed on the same day and at different days over a period of two weeks. The result revealed the precision with %RSD respectively for intra-day and inter day. Results were shown in tables 1 and 2. The typical chromatogram of nicotinic acid, folic acid and cyanocobalamin mixture was shown in figure 4.

The accuracy or percentage recovery of nicotinic acid, folic acid and cyanocobalamin was performed and the results were shown in table 3.

The stability testing for nicotinic acid, folic acid and cyanocobalamin was performed and standard deviation and relative standard deviation were calculated. The results were shown in table 4.

CONCLUSION

The validation study shows that the developed method is accurate, rapid, precise, reproducible and inexpensive with acceptable RSD (%) and standard deviations which make it versatile and valuable for simultaneous determination of vitamins in bulk and pharmaceutical dosage forms.

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