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Research Article

METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF B-GROUP VITAMINS AND ATORVASTATIN IN PHARMACEUTICAL SOLID DOSAGE FORM BY RP-HPLC

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ABSTRACT

A simple and sensitive RP-HPLC method was developed and Validated for the simultaneous determination of B-group vitamins like Pyridoxine hydrochloride (B_6), folic acid (B_9), Methylcobalamine (B_{12}) and Atorvastatin(ATO) in Atherochek Tablets. The Methylcobalamine (B_{12}) was determined separately because of its low concentration in tablets. RP-HPLC analysis was performed with a Waters, Alliance e2690 HPLC system, equipped with a UV/VIS Waters 2489 detector. The procedures for the determination of B_6, folic acid & Atorvastatin were carried out on a Inertsil ODS-3, C-18 (250x4.6mm, 5miron) column and the Detection wavelength at 254 nm for Atorvastatin, vit B_6, folic acid and 265 nm for Vit. B_{12}. The obtained results and statistical parameters for all the investigated vitamins of the B-group and Atorvastatin in Atherochek tablets were satisfactory. The methods were validated according to ICH guidelines.

Keywords: RP-HPLC, Pyridoxine hydrochloride, Folic acid, Methylcobalamine, Atorvastatin.

INTODUCTION

Atherochek is a unique combination of Lipid-lowering drug like Atorvastatin and homocysteine-lowering vitamins like Methylcobalamin, folic acid and pyridoxine HCL. Although a change in life-style is often the method of first choice for lipid lowering, lipid-lowering drugs, in general, help to control elevated levels of different forms of lipids in patients with hyperlipidemia. Statins like Atorvastatin lowers cholesterol, Homocysteine is a non-protein amino acid. It is a homologue of the amino acid cysteine, While detection of high levels of homocysteine has been linked to cardiovascular disease, lowering homocysteine levels may not improve outcomes.[1] Deficiencies of the vitamins folic acid (B_9), pyridoxine (B_6), or B_{12} can lead to high homocysteine levels.[2]Supplementation with pyridoxine, folic acid, B_{12} reduces the concentration of homocysteine in the bloodstream.[3-4] In short, decreases the level of vita B_6, B_9, B_{12} causes the homocysteine and due to homocysteine it causes the cardiovascular disease. Atorvastatin is chemically (3R, 5R)-7-[2-(4-Fluorophenyl)-5-isopropyl-3-phenyl-4-(phenyl carbamoyl)-1H-pyrrol-1-yl]-3,5-dihydroxy heptanoic acid. It is an inhibitor of 3-hydroxy 3-metylglutaryl-coenzyme A(HMG-Coa) reductase. This enzyme catalyses the conversion of HMG-Coa to mevalonate, an early rate limitating steps in cholesterol biosynthesis. Atorvastatin is administered 10 and 80 mg per day to reduce the raised lipid levels in patient with primary hyperlipidemia or combined hyperlipidemia[5-7].

Pyridoxine is one of the compounds that can be called vitamin B_6. Pyridoxine assists in the balancing of sodium and potassium as well as promoting red blood cell production. It is linked to cardiovascular health by decreasing the formation of homocysteine. Pyridoxine may help balance hormonal changes in women and aid the immune system.[8]

Vitamin B_{12} is an essential vitamin, required for DNA synthesis (and ultimately cell division) and for maintaining nerve myelin integrity.[9] It is found almost exclusively in animal-based products including red meats, poultry, seafood, milk, cheese and eggs.

Vitamin B_9 (folic acid and folate) is essential to numerous bodily functions. The human body needs folate to synthesize DNA, repair DNA, and methylate DNA as well as to act as a cofactor in certain biological reactions[10].It is especially important in aiding rapid cell division and growth,

such as in infancy and pregnancy. Children and adults both require folic acid to produce healthy red blood cells and prevent anemia.[11] structure of atorvastatin and vitamins explain in Fig.1.

RP-HPLC has been the analytical method of choice for the kinetic study of ATO and other Vitamins. several procedure of chromatographic techniques such as LC/MS/MS, HPLC with electorspray tandem mass spectrometry and LC methods with UV detector have been tested for the determination of ATO in biological fluid and pharmaceutical dosage forms.[12-15] However any generally recommended or rapid analytical method for the determination of ATO and Vitamins simultaneously and Validation has not yet described in any pharmacopoeia and literature. In the present study a new rapid, selective, linear, precise and sensitive RP-HPLC with UV detector was applied for the determination of ATO and other Vitamins in tablets.

MATERIAL AND METHOD

REAGENTS AND SOLVENTS

All chemicals and reagents used were of analytical grade and the water was distilled and filtered through a membrane filter. Pyridoxine hydrochloride (Vit. B_6), folic acid (Vit. B_9) and Metylcobalamine (Vit. B_{12}), Atorvastatin (ATO) were used as working standards. Methanol for HPLC, Phosphate Buffer, were used to prepare the mobile phase and Orthophosphoric acid used for adjusting the pH.

DOSAGE FORM

Atherochek tablets manufactured by Indoco Remedies Ltd. (Atherochek per tablet contains: Atorvastatin 5 mg, pyridoxine hydrochloride 10mg, folic acid 5mg and Metylcobalamine 500mcg).

CHROMATOGRAPHIC PARAMETERS FOR OF VITAMINS B_6, FOLIC ACID AND ATORVASTATIN

Equipment: High performance liquid chromatography equipped with Auto Sampler and UV detector.
Column: Inertsil ODS-3, C-18(4.6x250 mm, 5µm)
Flow rate: 1.0ml/min
Mobile phase: **A.** pH-3 Phosphate buffer.
B. 100% Acetonitrile.
Wavelength : 254nm
Injection volume: 25µl
Column oven: 30^{0}c
Run time: 20.0min
Gradient Programmed-Mention in Table 1

CHROMATOGRAPHIC PARAMETERS FOR VITAMINS B_{12}

Equipment: High performance liquid chromatography equipped with Auto Sampler and UV detector.
Column: Inertsil ODS-3, C-18(4.6x250 mm,5um)
Flow rate: 1.0ml/min
Mobile phase: Methanol
Wavelength: 265nm
Injection volume: 50µl
Column oven: 30^{0}c
Run time: 25 min

PREPARATION OF MOBILE PHASE

Mobile phase for the determination of vitamin B_6, B_9, and Atorvastatin

Mobile phase A: Phosphate buffer pH 3.Added 1 ml of ortho-phospheric acid in 1000 ml of water and adjust the pH 3 with 10% Potassium Hydroxide solution.

Mobile phase B: 100% Acetonitile.

Diluent: Prepared a mixture of Phosphate buffer pH 3.0 & Acetonitrile in 50:50 v/v ratio. Mixed above mixture and Dimetylformamide in 95:5 v/v ratio & used as diluents.

Mobile phase for the determination of vitamin B_{12}

100%Methanol.

METHOD VALIDATION

The method was validated for linearity, accuracy, intra-day and inter-day precision and robustness, in accordance with ICH guidelines[16].

Precision

One set of three different concentrations of mixed standard solutions of ATO and vit.B_6, vit.B_9 were prepared. All the solutions were analyzed thrice, in order to record any intra day variations in the results. For Inter day variations study three different concentrations of the mixed standard solutions in linearity range were analyzed on two consecutive days. One set of vit.B_{12} were prepared and analyzed thrice, in order to record any intra day variations in the results. The peak areas were recorded and Relative standard deviation (RSD) was calculated for both series of analyses.

Linearity

Aliquots 0.5, 1, 2, 3 and 4 mL of stock solution of ATO and 1, 2, 4, 6 mL working standard solution of vit.B_6, 2,4,5,6, and 8 mL, of vit.B_9, and 0.5,1,2,3,4 mL of vit.B_{12} were transferred in a series of 20 mL. for vit.B_{12} in 50 mL in calibrated volumetric flasks. Five replicates per concentration were injected and chromatograms were recorded. The peak area for ATO, vit.B_6, vit.B_9, vit.B_{12} were

calculated and respective calibration curves were plotted of response factor against concentration of each drug.

Accuracy

To check the accuracy of the method, recovery studies were carried out by addition of standard drug solution to pre-analyzed sample solution at three different levels 50%, 100% and 150%. The percentages of recoveries were calculated, results of which are represented in Table 2 and 3

Robustness

In the robustness study, the influence of small, deliberate variations of the analytical parameters on retention time of the drugs was examined. The following two factors were selected for change: flow rate of the mobile phase (1.0 ± 0.02 ml/min), a wavelength at which the drugs were recorded (254 ±2 nm) for vit. B_6, vit.B_9, ATO and (265± 2 nm) for vit. B_{12}.One factor at the time was changed to estimate the effect. The solutions of 100ppm for ATO, 200ppm for vit. B_6, 100ppm for vit. B_9, 20ppm for vit. B_{12} were applied onto the column. A number of replicate analyses (n = 3) were conducted at three levels of the factor (-, 0, +). It was observed that there were no marked changes in the chromatograms, which demonstrated that the RP-HPLC method developed is robust.

RESULTS & DISSCUSSION

For RP-HPLC method different mobile phases were tried and the mobile phase containing Acetonitrile and phosphate buffer pH 3 was found to be optimal for obtaining well defined and resolved peaks with mean retention times 2.358 min for vit.B_9, 1.952 min for vit.B_6 and 10.837 min for ATO. 2.516 min, for vit. B_{12}, Results were found to be linear in the concentration range 25-250ppm for ATO, 50-500 for vit. B_6, 10-100ppm for vit. B_9, 2-50ppm for vit. B_{12}.The correlation coefficients for the plots were 0.999 for AT0 and 0.999 for vit. B_6. 0.999 for vit. B_{12}, 0.998 for vit. B_9. The proposed method was also evaluated by the assay of commercially available tablets containing ATO and vit. B_6.vit. B_{12}, vit. B_9.The method was found to be accurate and precise, as indicated by recovery studies and % RSD not more than 2. Robustness of the method, checked after deliberate alterations of the analytical parameters shown no marked changes in the chromatograms (RSD < 2), which demonstrated that the RP-HPLC method developed is robust. The summary of validation parameters of proposed HPLC method is given in Table 4. The representative chromatogram of the standard solution of mixture is shown in Fig. 2 and 3.

CONCLUSION

The validated RP-HPLC method employed here proved to be simple, fast, accurate, precise and robust, thus can be used for routine analysis of vit. B_6, vit. B_9, vit. B_{12} and ATO in combined dosage form.

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Table 1: Gradient Programmed

Time(Min.)	Mobile Phase-A (%)	Mobile Phase-B (%)
0.0	60	40
5.0	60	40
7.0	20	80
9.0	0.0	100
13.0	0.0	100
15.0	50	50
20.0	60	40

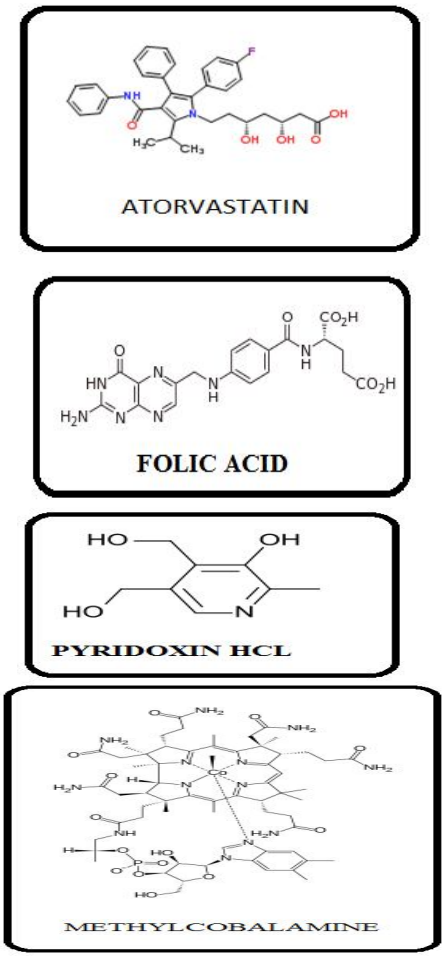


Fig. 1: Structure of Atorvastatin and Vitamins

Table 2: Accuracy of ATO and vit.B6

Level of % Recovery	% Mean Recovery[a]		Standard Deviation		% R.S.D.[a]	
	ATO	vit.B_6	ATO	vit.B_6	ATO	vit.B_6
50	49.64	50.64	0.129	0.340	0.260	0.672
100	101.14	101.02	0.268	0.303	0.265	0.300
150	149.74	150.20	0423	0.182	0.282	0.121

*Avg. of three determinations, R.S.D. is relative standard deviation

Table 3: Accuracy of vit.B9 and vit.B12

Level of % Recovery	% Mean Recovery[a]		Standard Deviation		% R.S.D.[a]	
	vit.B_9	vit.B_{12}	vit.B_9	vit.B_{12}	vit. B_9	vit.B_{12}
50	50.20	50.56	0.053	1.250	0.106	0.143
100	101.22	101.66	0.034	1.580	0.033	0.140
150	150.67	149.66	0.301	2.157	0.200	0.157

*Avg. of three determinations, R.S.D. is relative standard deviation

Table 4: Summary of validation parameters of proposed RP-HPLC method

Parameters	ATO	vit B_6	vit B_9	vit B_{12}
Linearity range (ppm)	25-250	50-500	10-100	2-50
Correlation co-efficient	0.999	0.999	0.998	0.999
Accuracy (% Recovery)				
50%	49.64%	50.64%	50.20%	50.56%
100%	101.14%	101.02%	101.22%	101.66%
150%	149.74%	150.20%	150.67%	149.96%
Precision (% RSD)[c]				
Intraday (n[d] = 3)	0.10	1.87	0.57	0.27
Inter day (n = 3)	0.26	0.24	0.40	0.10

[c]RSD = Relative standard deviation.
[d]n = Number of determination

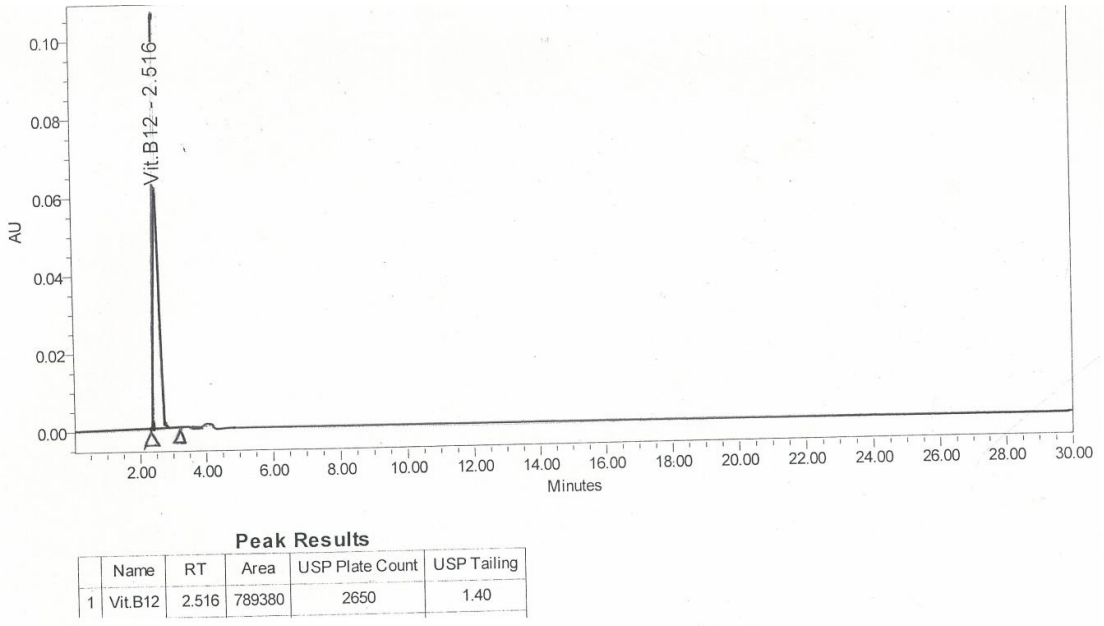
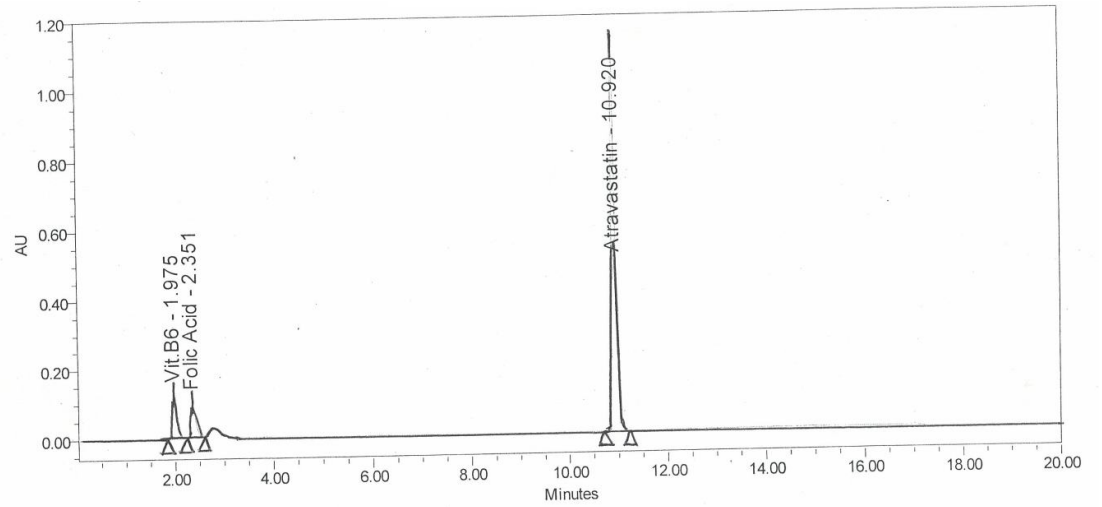


Fig. 2: Representative Chromatogram obtained for standard containing vit.B12 (20 ppm, 2.516 min)



Peak Results

	Name	RT	Area	USP Plate Count	USP Tailing	Resolution
1	Vit.B6	1.975	871811	2067	1.31	
2	Folic Acid	2.351	673502	2555	1.51	2.04
3	Atravastatin	10.920	6209707	94919	1.30	54.22

Fig. 3: Representative chromatogram obtained for standard mixture containing ATO (100ppm, 10.920 min), vit.B_9 (100 ppm, 2.351 min) and vit.B_6 (200ppm, 1.975min)

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