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RP-HPLC Method Development and Validation for Simultaneous Estimation of Amitriptyline Hydrochloride and Methylcobalamin in Bulk and Tablet Dosage Form

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ABSTRACT:A simple, novel, precise, and cost-effective reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the simultaneously estimation of amitriptyline HCl and methylcobalamin in bulk and the marketed tablet dosage form. The chromatographic separation was carried out on a Shimadzu C18 (150 x 3 mm, 3 µm) column using a mobile phase of water: methanol(70:30 v/v). The flow rate was 0.7 ml/min with detection at 265 nm using UV detector. The retention time was found to be 0.930 min for Amitriptyline HCl and 2.775 min for Methylcobalamin. The method were validated by specificity ,linearity ,accuracy, precision, limit of detection, limit of quantification and robustness.The linearity was obtained in the concentration range of 10-50 µg/ml for Amitriptyline HCl and 5-25 µg/ml for Methylcobalamin .The correlation coefficient ('r2' value) for Amitriptyline HCl and Methylcobalamin was 0.9978 and 0.9988, respectively. The percentage recoveries ofAmitriptyline HCl and Methylcobalamin was found to be in the range of99.0-100.9% and 99.6 -101.2% respectively.LOD and LOQ were found to be 2.8 µg/mland 8.5 µg/ml for Amitriptyline HCl 1.0 µg/ml and 3.1 µg/ml for Methylcobalamin.A simple and precise method was developed for the assay of Amitriptyline HCl and Methylcobalamin from tablet formulation. The method does not require the use of expensive reagent and also less time consuming, hence it can be used for the routine analysis ofAmitriptyline HCl and Methylcobalamin in tablet dosage form.

KEYWORDS: Amitriptyline HCl, Methylcobalamin,Methanol,Reversed Phase HPLC.

I. INTRODUCTION:

Amitriptyline is an antidepressant with sedative effects.Amitriptyline inhibits the membrane pump mechanism responsible for uptake of nor-epinephrine and serotonin in adrenergic and serotonergic neurons.Amitriptyline is chemically N,N-dimethyl-3-(2-tricyclo[9.4.0.03,8] pentadeca 1(15),3,5,7,11,13hexaenylidene)propan-1-amine. Methylcobalamin is chemically Carbanide;cobalt(2+);[(2R,3S,4R,5S)-5-(5,6-dimethylbenzimidazole-1-yl)-4)hydroxyl 2(hydroxyl methyl)Oxolan-3-yl]1[3[1R,2R,3R,5Z,12S,13S,15Z,17S,18S,19R)-2,13,18,-tris(2-ammino-2-oxoethyl)-7,12,17-tris(3-amino-3-oxopropyl)-3,5,8,8,13,15,18,19-octamethyl-2,7,12,17-tetrahydro-H-corrin-24-id-3-yl]propanoylamino]propane-2-yl hydrogen phosphate,VitaminB12 is used in the treatment of pernicious anaemiaand other vitamin B12 eficiency states.Amitriptyline and Methylcobalamain are used in combination belongs to a group of medication known as central nervous system agents used to manage neuropathic pain due to damaged nerves especially caused by a lesion or disease of somatosensory nervous system.Several spectroscopic HPTLC, HPLC methods has been used for the analysis of Amitriptyline HCl and Methylcobalamin in individual dosage form and in combination with other drugs,but none of the method were reported for combination of these drugs,therefore the thought of cost-effective rapid RP-HPLC method development and simultaneous estimation of Amitriptyline HCl and Methylcobalamin in tablet dosage form.

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Figure no1:Structure of Amitriptyline HCl

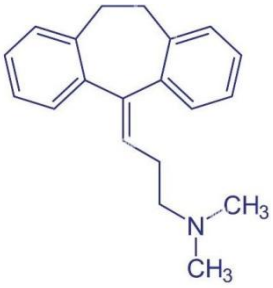
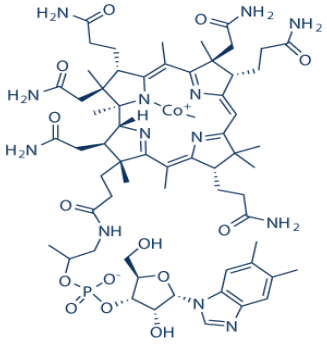


Figure no: 2 Structure of Methylcobalamin



II. MATERIALS AND METHOD:

Instrumentation:Chromatography was performed on Shimadzu prominence –LC-20AR system with lab solution software for data processing. Separationand quantitation were made on shimadzu C18column (150×3nm,3µm).

Chemicals and Reagents: Amitriptyline HCl (99.8) and Methylcobalamin (99.10) reference standards were gift sample by madras pharmaceuticals,Chennai. Methanol HPLC grade (High media),Water HPLC grade(Thermo fisher scientific,Mumbai.were used for preparing mobile phase and stock solution.

Preparation of Mobile Phase: 70 volumes of HPLC grade water and 30 volumes of HPLC grade methanol were used as the mobile phase.

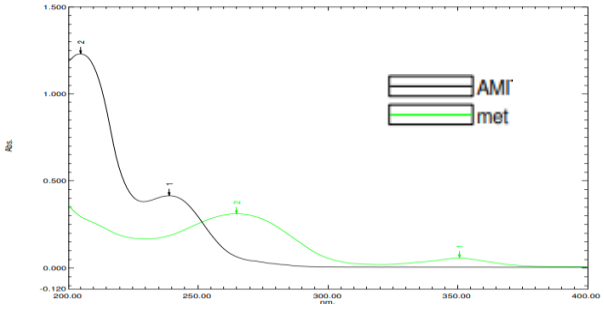
Preparation of Diluent: Based on the Solubility of drug, diluent was selected as Water:Methanol (70:30).

Preparation of Standard Stock Solution: About 10 mg of each of reference standard of amitrypitiline HCl and methylcobalamin was weighed accurately and transferred to two separate 10 ml volumetric flask. Both drugs were dissolved in water to get a concentration of 1000 µg/ml. From the above solution pipette out 1 ml and transferred to 10mlwith methanol to get a concentration of 100 µg/ml. From the stock solution, mixed standard solution was prepared to contain 10 µg/ml of Amitriptyline HCl and 5 µg/ml of methylcobalamin

Preparation of sample solution: Ten tablets each containing 10mg of amitriptyline HCl and 1.5mg of methylcobalamin were weighed and finely powdered; a quantity of powder equivalent to 10 mg of amitriptyline HCl and 10 mg of methylcobalamin was weighed and transferred to 100ml volumetric flask and the drugs were extracted with diluent in ultrasonicator for 30 mins and made up to 100 ml with the diluent. The solution was filtered using Whatman filter paper and the filtrate solution is used as sample solution. 1ml of this solution was pipetted in to 10 ml and made upto volume with diluent.

Selection of Wavelength: Standard solution of Amitriptyline HCl and Methylcobalamin was scanned using a double beam UV visible spectrophotometer between the range 200nm to 400nm, and overlain spectra were obtained. The wavelength selected was 265nm, which is an isobestic point. The overlay spectrum of Amitriptyline HCl and Methylcobalamin are shown in **figureno:3**

Figure 3: Overlay Spectrum



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Chromatographic Condition: Method was developed using a Shimadzu C18 (150 x 3 mm, 3 µm) column using a mobile phase of water: methanol(70:30 v/v) at flow rate of 0.7 ml/min . Detection wavelength 265nm was selected by scanning standard drug solution over a wide range of wavelengths 200-400 nm using a spectrometer. The injection volume was 20µl.

Method validation:

The developed RP- HPLC method has been validated according to the guidelines of ICH. The validated parameters were linearity and range,accuracy,precision,, robustness,LOD and LOQ. For all the parameters %RSD were calculated.

Specificity : Specificity of the method can be termed as the absence of any interference at a retention time of samples during the analysis. Specificity of the method was performed by injecting blank and standard drug preparations. Chromatograms were recorded and retention times fromstandard and sample preparations were compared for identification of analytes present.

Linearity : A series of standard solutions 10-50 µg/ml of Amitriptyline HCl and 5-25 µg/ml of Methylcobalamin were prepared. 20µl of each standard solutions was injected 3 times and peak area was observed. The linearity graph was a plot of peak area versus the concentration. From this, the correlation coefficient and regression equation were generated to find out the unknown concentration of sample. The calibration data of Amitriptyline HCl and Methylcobalamin is given in Table 2& 3, Figure no: 6and 7represent linearity graphs of both drugs respectively.

Accuracy : Accuracy of the method is the closeness of test results obtained by the Accuracy determined over the range of 80%,100% and 120% of the sample concenteration. The recovery of an analytical method is determined by applying the method to analyze samples to which known amounts of analyte have been added. The recovery is calculated from the percentage of analyte recovered by the assay. The known amounts of standard solutions of amitriptyline HCl (8, 10 and 12 µg/ml) and methylcobalamin (8, 10 and 12 µg/ml) were added to a pre quantified test solutions of amitriptyline HCl (10 µg/ml) and methylcobalamin (10 µg/ml). Each solution was injected in triplicate, and the recovery was calculated by peak areas and % RSD was noted. Results are shown in table 4.

Precision: The method was validated regarding intra-day and inter-day precision. The intra-day and inter-day study were performed by injecting 10, 30 and 50 µg/ml of Amitriptyline HCland 5, 15and 25 µg/ml of Methylcobalamin solutions three times for each aliquot. The % RSD for the precision study was found less than 2% as shown in table 5,6 and 7.

Limit of detection(LOD):

The limit of detection in the smallest concentration can be detected and not quantified as an exact value.LOD can be calculated as

$$LOD=3.3\sigma/S$$

Where σ=standard deviation of the y –intercept ,
S= slope of calibration curve.

Limit of quantification(LOQ)

The limit of the quantification is the lowest amount of analyte in the sample which can be determined quantitatively.

$$LOQ=10\sigma/S$$

Where σ=standered deviation of the y-intercept,
S=slope of calibration curve.

Robustness:

Robustness of the method was performed by variation in the flow rate and temperature during the analysis. The difference has been made to the analytical method in order to check and eveluvate the capacity of the newly developed method, how much it remain unaffected by such variations. An aliquot of standard solutions were injected in triplicate. The results show that percentage relative standard deviation is not more than 2.0%.

III. RESULTS AND DISCUSSION:

In the current method, the chromatographic conditions were optimized to obtain complete elution of Amitrypitiline HCl and Methylcobalamin. Mobile phase, its ratio and flow rate selection was based on resolution between drug peaks , peak parameters such as height, tailing factor, theoretical plates and total run time. The run time was set at 10 min and the retention time for Amitrypitiline HCl and Methylcobalamin was found 0.93min and 2.77 min as shown in Figure 6 and 7. 20µl of standard and sample solutions was injected 6 times and compared the retention times and were found to be same. The regression equation was used to estimate the

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amount of Amitrypitiline HCl and Methylcobalamin, either in formulation and in validation study (precision and accuracy). Robustness of the developed method was determined by analysis of sample by making changes in various parameter like flow rate, and temperature using similar operationaland environmental conditions.

Chromatographic separation was achieved by using shimadzu C18 (150 x3mm,3 µm) column with water and methanol in the ratio of (70:30) as the mobile phase at the flow rate of 0.7ml/min and detection was carried at 265 nm. The optimization and method development resulted in the elution of amitriptyline at0.93min and methylcobalamine at 2.77 min the total run time was 10mins.

Optimized condition:

The optimized condition for the chromatagram include pump mode,column,mobile phase,flow rate injection volume, detection wavelength and retention time are clearly mentioned in the below table no: 1 along with their specifications.

Table no: 1 Optimized Chromatographic Condition

Parameters	Optimized Conditions
Pump mode	Binary
column	Shimadzu C18
Mobile phase	Water:Methanol (70:30 V/V)
Flow rate	0.7ml/min
Injection volume	20µl
Detection wavelength	265nm
Retention time	0.937&2.77min

The developed RP-HPLC method for Amitriptyline HCl and Methylcobalamin was validated as per ICH guidelines.

Specificity:

Specificity of an analytical method can be demonstrated through absence of interference.Method specificity was determined by observing and comparing the test result obtained for the sample solution with the standard result obtained for a pure drug. The standard drug and sample chromatogram is shown in figure no: 4&5

Figure no: 4 Chromatogram of Standard

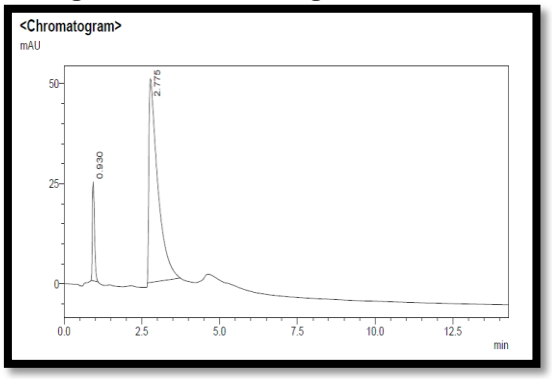

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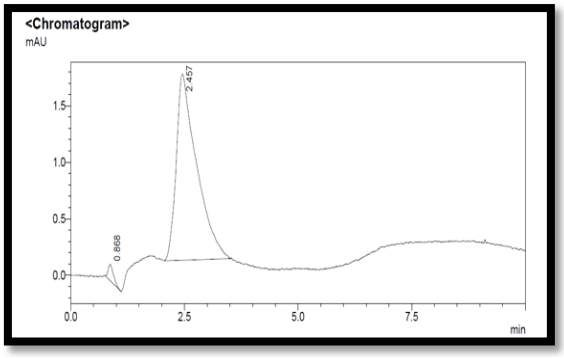
Figure no: 5 Chromatogram of Sample solution



Linearity The linearity of the method was determined at five concentration levels ranging from 10-50 µg /ml for Amitriptyline HCl and 5-25 µg/ml for Methylcobalamin. The linearity graph was constructed by plotting peak areas against concentration of drugs. The slope and intercept value for calibration curve was y = 2731.8x + 10913 (R^2 = 0.9978) for Amitriptyline HCland y = 5555.1x+65220(R^2 = 0.9988) for Methylcobalamin. The results show that an excellent correlation exists between the peak area and concentration range mentioned above (table no: 2&3).

Table no:2 Linearity of Amitriptyline HCl

	Amitriptyline HCl	
	Concentration (µg/ml)	**Peakarea**
1	10	36688
2	20	66530
3	30	93143
4	40	122857
5	50	145113
Correlation coefficient value		**0.9978**

Table no: 3 Linearity of Methylcobalamin

	Methylcobalamin	
S.No	**Concentration (µg/ml)**	**Peakarea**
1	5	91612
2	10	121045
3	15	150907
4	20	176317
5	25	202854
Correlationcoefficient value		**0.9988**

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Figure no: 6 Linearity graph of Amitriptyline HCl

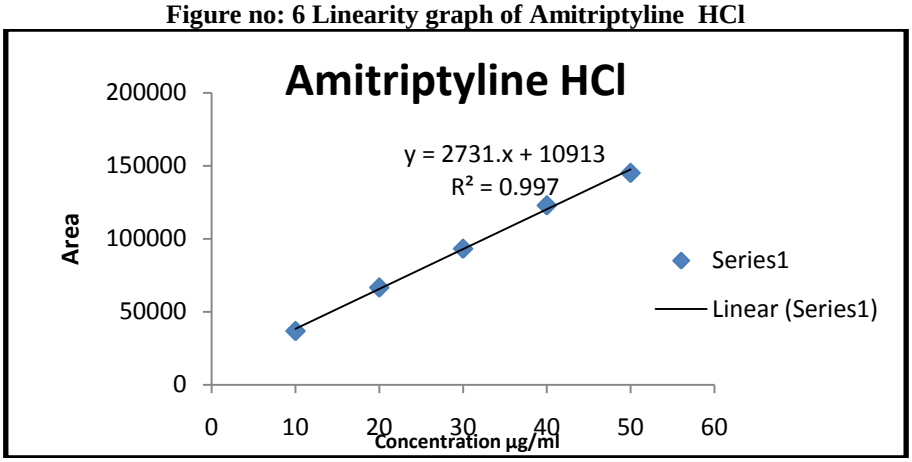
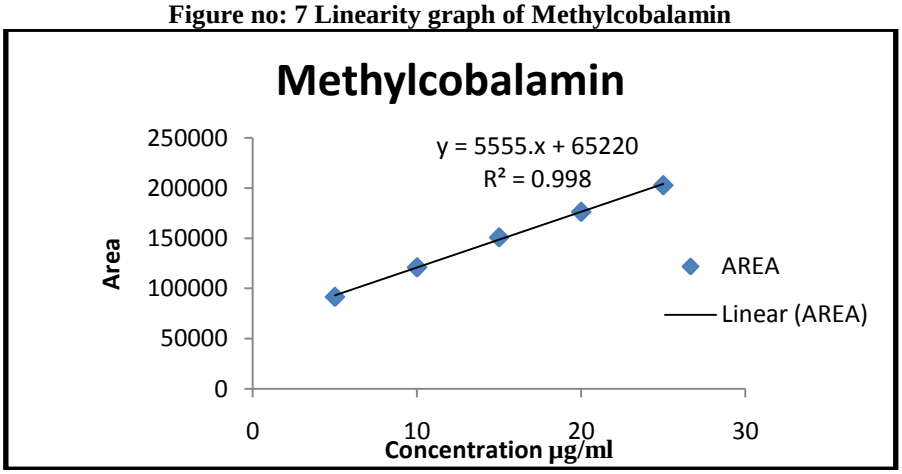


Figure no: 7 Linearity graph of Methylcobalamin



Accuracy: Accuracy of the method was confirmed by recovery study at three levels (80%, 100%, and 120%) of standard addition. Percentage recovery for Amitriptyline HCl was found to be 99.40%to 100.92%, while for Methylcobalamin it was found to be 99.61% to 101.27% as shown in table no: 4

Table no: 4 Recovery Studies

S.No	Level	%Recovery		%RSD	
		AMI	METCBN	AMI	METCBN
1	80%	99.40	99.61	1.39	0.86
2	100%	100.92	100.32	1.21	0.59
3	120%	99.01	101.27	0.74	1.59

*%RSDof three determinations

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Presicion: The % RSD for repeatability study for Amitriptyline HCl and Methylcobalamin was found to be 1.47 and 1.20 respectively. The Inter-day and Intra-day study also show % RSD value for Amitriptyline HCl and Methylcobalamin within the acceptable limit. Results for precision study are shown in table no: 5,6 and 7.

Table no: 5 Intraday precision

Level	Concentration(µg/ml)		Peakarea		%RSD	
	AMI	METCBN	AMI	METCBN	AMI	METCBN
I	10	5	36688	91612	0.421	0.536
			36479	92578		
			36780	91925		
II	30	15	93143	150907	0.794	0.623
			92849	151834		
			94255	152799		
III	50	25	145113	202454	0.719	0.716
			144609	201906		
			143119	199730		

*%RSDof three determinations

Table no: 6 Interday presicion

Level	Concentration(µg/ml)		Peakarea		%RSD	
	AMI	METCBN	AMI	METCBN	AMI	METCBN
I	10	5	36688	91612	1.076	1.253
			37479	92578		
			37180	93925		
II	30	15	93143	150907	0.938	1.329
			93949	154834		
			94905	153799		
III	50	25	145113	202454	1.581	1.431
			147509	202906		
			142919	207730		

*%RSDof three determinations

Table no: 7 Repeatability

Concentration(µg/ml)		Peakarea		%RSD	
AMI	METCBN	AMI	METCBN	AMI	METCBN
10	10	36688	121025	1.47	1.20
		36502	119158		
		35689	123162		

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		35701	119965		
		36226	120325		
		36028	121985		

*%RSDof six determinations

LOD and LOQ : The LOD was found to be 2.8 µg/ml for AmitrypitilineHCl and 1.0 µg/ml for Methylcobalamin, while the LOQ was found to be 8.5 µg/ml for AmitrypitilineHCl and 3.1 µg/ml for Methylcobalamin shown in table no:8.

Table no: 8 LOD and LOQ

Drug	LOD	LOQ
AmitryptylineHCl	2.8 µg/ml	8.5 µg/ml
Methylcobalamin	1.0 µg/ml	3.1 µg/ml

Analysis of tablet by proposed method: Applicability of the proposed method was tested by analyzing the commercially available marketed formulation. The percentage of Amitriptyline HCl and Methylcobalamin were found to be 99.91 % and 98.13% respectively. Results as % assay are shown in table no: 9.

Table no: 9 Analysis of formulation

Drug	Labelled amount	Amount found	% Assay	%RSD
Amitriptyline HCl	10mg	9.99 mg	99.91	0.53
Methylcobalamin	1.5mg	1.47 mg	98.13	0.41

*%RSDof three determinations

IV. CONCLUSION:

The proposed RP-HPLC method was found to be simple, specific, precise, accurate, rapid and economical for simultaneous estimation of Amitriptyline HCl and Methylcobalamin in combined tablet dosage form. The developed method has been validated according to guidelines of ICH . The Validation parameters was found to be within limit and %RSD was less than 2%. Analysis of formulation shows good agreement with their respective label claims. Hence, this method can be easily and conveniently adopted for routine analysis of Amitriptyline HCl and Methylcobalamin in combined tablet dosage form.

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