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Research Article

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS QUANTITATIVE ESTIMATION OF PREGABALIN, MECOBALAMIN AND ALPHA LIPOIC ACID IN CAPSULES

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ABSTRACT

Objective: To develop a simple, precise, accurate, linear and rapid Reverse Phase High Performance Liquid Chromatographic (RP-HPLC) method for the simultaneous quantitative estimation of Pregabalin 75mg, Mecobalamin 750µg and Alpha lipoic acid 100mg in capsules as per ICH guidelines.

Methods: The optimized method uses a reverse phase C18 column, Enable Make C18G (250 X4.6mm, 5µm), mobile phase of a mixture of potassium dihydrogen orthophosphate buffer (20mM, pH adjusted to 6 using 0.1N potassium hydroxide solution), methanol and acetonitrile in ratio of 75:10:15v/v, flow rate of 1.2 ml/min and a detection wavelength of 210 nm using a UV detector.

Results: The developed method resulted in pregabalin eluting at 2.6 min, mecobalamin eluting at 6.4 min and alpha lipoic acid eluting at 11.3 min. The method exhibited linearity over the range of 187.5-750µg/ml, 1.87-7.5µg/ml and 250-1000µg/ml for pregabalin, mecobalamin and alpha lipoic acid respectively. The method precision is exemplified by relative standard deviations of 0.84% for pregabalin, 1.07% for mecobalamin and 0.85% for alpha lipoic acid. Accuracy studies revealed % mean recoveries during spiking experiments between 98 and 102. The limit of detection was obtained as 3µg/ml for pregabalin, 800ng/ml for mecobalamin and 500ng/ml for alpha lipoic acid, while the limit of quantitation was obtained as 6 µg/ml for pregabalin, 2µg/ml for meocobalamin and 1.5µg/ml for alpha lipoic acid.

Conclusion: A simple, precise, accurate, linear and rapid RP-HPLC method was developed and validated as per ICH guidelines and hence can be applicable in routine analysis for tablets in various pharmaceutical industries.

Keywords: RP-HPLC, Pregabalin, Mecobalamin, Alpha lipoic acid, Validation.

INTRODUCTION

Alpha Lipoic acid (**Figure 1**) (6,8-thioctic acid, or 1,2-dithiolane-3-pentanoic acid, or 1,2-dithiolane-3-valeric acid) is a natural antioxidant present in prokaryotic and eukaryotic cells. Alpha lipoic acid supplementation is advocated in the treatment of AIDS, Chaga, diabetes, heavy metal poisoning, ischemia-reperfusion injury, liver diseases (alcoholic liver disease, mushroom poisoning), neurodegenerative disorders, radiation injury, Wilson's disease and the effect of cigarette smoking [1-2]. Methylcobalamin (**Figure 2**) is a form of vitamin B12 used in the treatment of trigeminal neuralgia, megaloplastic anemia, diabetic neuropathy and facial paralysis in Bell's pasly syndrome. It is chemically Coα-[α-(5,6-dimethylbenz-1H-imidazolyl)]-Coβmethylcobamide [3-8]. Pregabalin (**Figure 3**) [(*S*)-3-(amino methyl)-5-methyl hexanoic acid] is an anticonvulsant drug used for neuropathic pain and as an adjunct therapy for partial seizures with or without secondary generalization in adults [9,10]. Pregabalin binds with high affinity and specificity to voltage-gated calcium channel alpha (2)-delta proteins [11,12].

Fig. 1: Structure of Alpha lipoic acid

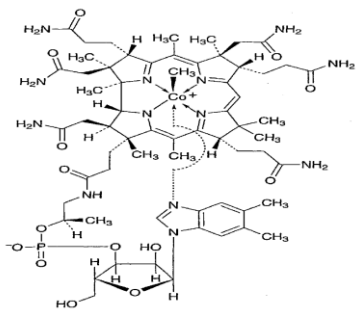

Fig. 2: Structure of Methylcobalamin

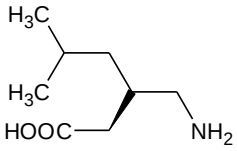

Fig. 3: Structure of Pregabalin

A detailed literature survey reveals various RP-HPLC analytical methods for the quantitative estimation of Alpha lipoic acid [13,14], Pregabalin [15,16], Mecobalamin [17] individually in bulk, plasma and in various pharmaceutical dosage forms. RP-HPLC methods are reported for the analysis of combination of various drugs with alpha lipoic acid [18-21], similarly pregabalin with other drugs [22,23] and Mecobalamin with other drugs [22,23]. As per our detailed literature survey as on date, there are no RP-HPLC methods available for simultaneous quantitative estimation of Pregabalin, Mecobalamin and Alpha lipoic acid in any matrix either of pharmaceutical dosage forms, plasma, etc. In addition there exist no pharmacopeial methods available for analysis of these three drugs in combination. Thus, we here report a simple, precise, accurate, linear and a convenient RP-HPLC method and validation as per ICH guidelines [24] for simultaneous quantitative estimation of Pregabalin, Mecobalamin and Alpha lipoic acid in capsules.

MATERIALS AND METHODS

Chemicals and Reagents

Analytically pure samples of Pregabalin, Mecobalamin and Alpha lipoicacid with purities greater than 99% were obtained as gift samples from Biophore and Chandra labs (Hyderabad, India) and capsule formulation [NERVUP PG (Brand name)] was procured from Medplus pharmacy, Hyderabad, India with labelled amount of Alpha lipoic acid 100 mg, Mecobalamin 750µg and Pregabalin 75mg. Acetonitrile and Methanol (HPLC grade) were obtained from Sigma Aldrich (Hyderabad, India), water (HPLC grade), potassium

dihydrogen orthophosphate (AR grade) and potassium hydroxide (AR Grade) were obtained from SD Fine chemicals (Hyderabad, India). 0.45µm Nylon membrane filters were obtained from Spincotech private limited (Hyderabad, India).

Instrument

HPLC analysis was performed on Shimadzu LC-20AD Prominence Liquid Chromatograph comprising a LC-20AD pump, Shimadzu SPD-20A Prominence UV-Vis detector and Enable Make C18G reverse phase column (250X4.6 mm, 5µ). A manually operating Rheodyne injector with 20 µL sample loop was equipped with the HPLC system. The HPLC system was controlled with "Lab solutions lite" software. In addition, an electronic analytical weighing balance (0.1mg sensitivity, Shimadzu AY 220), digital pH meter (DELUX model 101), a sonicator (sonica, model 2200 MH) and UV-Visible Spectrophotometer (Shimadzu UV-1800 series, software:UV probe version 2.42) were used in this study.

Method

Selection of Wavelength

Suitable wavelength for the HPLC analysis was determined by recording UV spectrums in the range of 200-400 nm for individual drug solutions of Pregabalin, Mecobalamin and Alpha lipoic acid. Suitable wavelength selected was 210 nm (**Figures:4-6**).

Chromatographic conditions

The separation of the drugs was achieved on a Reverse phase C18 column, Enable Make C18G (250X4.6 mm, 5µ). The mobile phase consists of a mixture of potassium dihydrogen orthophosphate buffer (20mM, pH adjusted to 6 using 0.1N potassium hydroxide), methanol and acetonitrile in ratio of 75:10:15, v/v. The mobile phase was set at a flow rate of 1.2 ml/min and the volume injected was 20 µl for every injection. The detection wavelength was set at 210 nm.

Buffer Preparation

The buffer solution is prepared by weighing 2.736g of potassium dihydrogen orthophosphate (KH_2PO_4) and transferring to 1000 ml of HPLC grade water to get 20 mM buffer strength and later pH was adjusted to 6 using 0.1N potassium hydroxide solution. The buffer was then filtered through 0.45 µm nylon membrane filter.

Mobile phase Preparation

The mobile phase was prepared by mixing buffer, methanol and acetonitrile in the ratio of 75:10:15, v/v and later sonicated for 10 minutes for the removal of air bubbles.

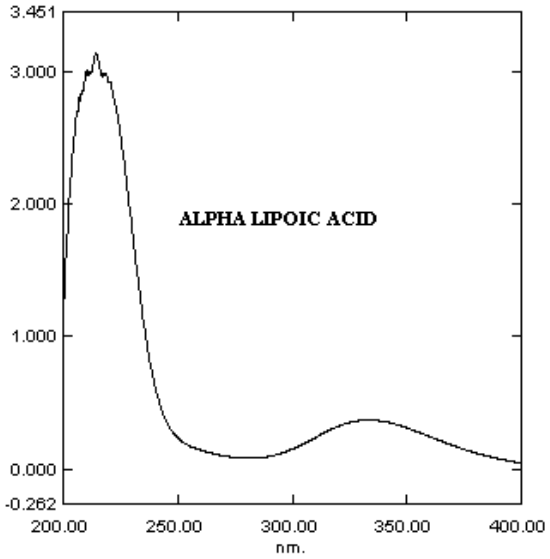

Fig. 4: UV spectrum of Alpha lipoic acid

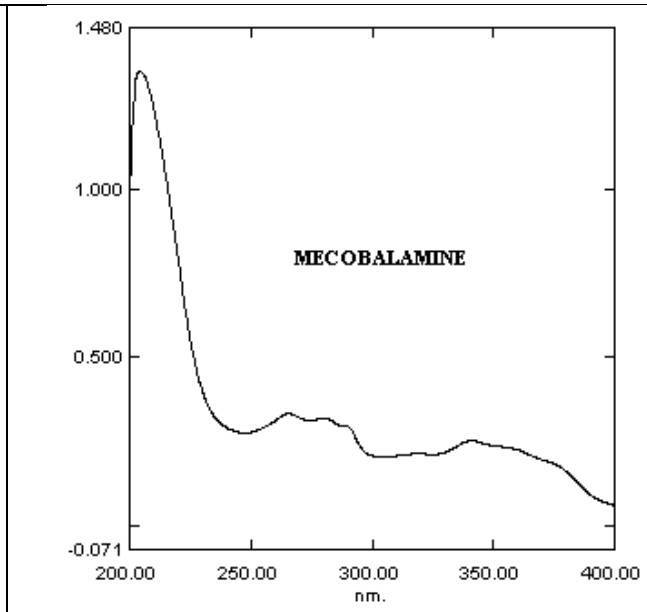

Fig. 5: UV spectrum of Mecobalamin

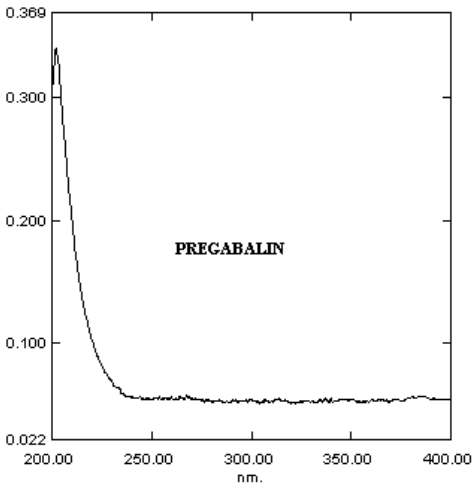

Fig. 6: UV spectrum of Pregabalin

Preparation of stock and working standard solution

75mg of Pregabalin, 750µg of Mecobalamin and 100mg of Alpha lipoic acid were accurately weighed and taken in 100 ml clean and dry volumetric flask containing 50 ml of diluent and then sonicated for 5 minutes to dissolve. Later the solution was made up to the mark using the mobile phase. This is considered as standard stock solution. 5ml of the stock solution was pipetted out and made up to 10ml to get working standard solution, treated as 100% target concentration.

Preparation of stock and working sample Solution

Sample solution containing three drugs was prepared by dissolving capsule powder into diluent (mobile phase). Ten capsules were emptied and then weighed separately and their average weights were determined. The average weight was weighed from the emptied ten capsules grinded in a pestle and mortar and then transferred to a 100 ml volumetric flask containing 80ml diluent. Sonication was done for five minutes and later the volume was made up to 100ml using mobile phase. Then the sample preparation was filtered through 0.45µm nylon membrane filter to get sample stock solution. 5ml of the stock solution was pipetted out and made up to 10ml to get working sample solution, treated as 100% target concentration.

RESULTS AND DISCUSSION

Method Development

A Reverse phase HPLC isocratic method was developed keeping in mind the system suitability parameters i.e. resolution factor (Rf) between peaks, tailing factor (T), number of theoretical plates (N), runtime and the cost effectiveness. The optimized method developed resulted in the elution of Pregabalin at 2.6 min, Mecobalamin at 6.4 min and Alpha lipoic acid at 11.3 min. **Figure 7** and **Figure 8** represent chromatograms of blank solution and mixture of standard solutions respectively. The total run time is less than 12 minutes. System suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time (*Rt*), number of theoretical plates (*N*), peak resolution (Rf) and peak Tailing factor (T) were evaluated for six replicate injections of the standards at working concentration. Results given in **Table 1** were within acceptable limits.

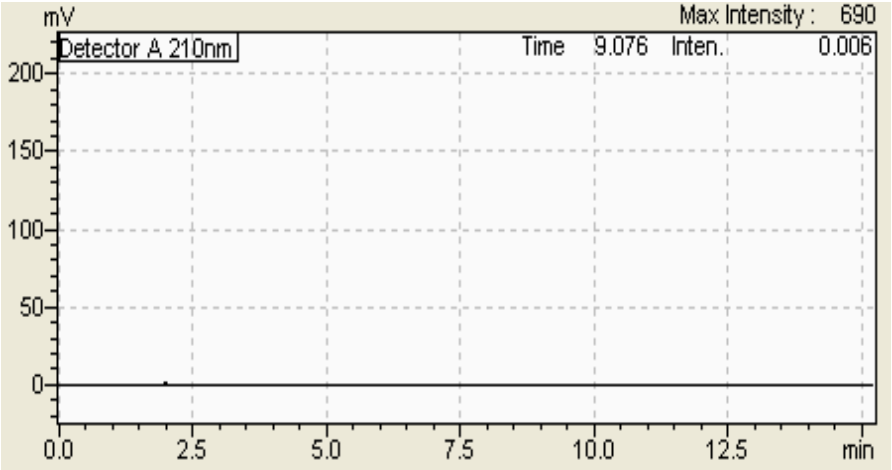

Fig. 7: Typical Chromatogram of Blank solution

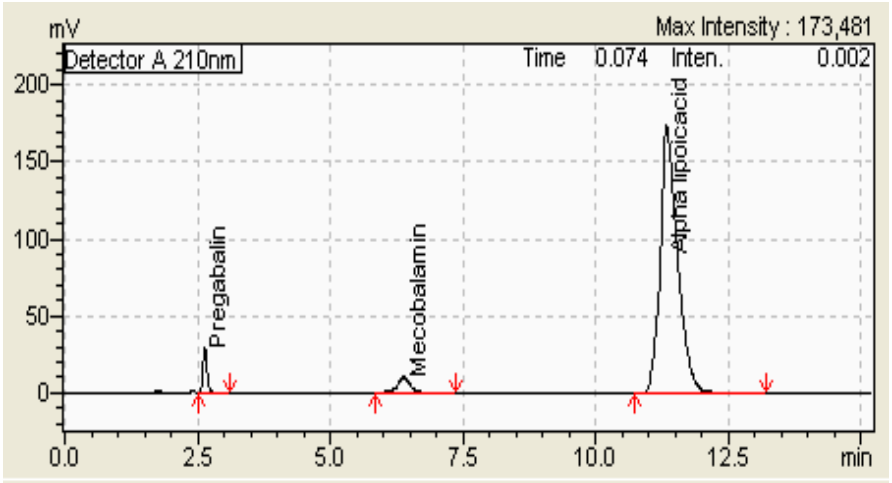

Fig. 8: Typical chromatogram for the mixture of Standard solutions

Table 1: System suitability studies results.

*Parameters	Required Limits	Pregabalin	Mecobalamin	Alpha lipoic acid
Retention time (min)	% RSD < 1%	2.6	6.4	11.3
Resolution factor (Rf)	Not less Than 2	13.25		10.04
Number Of Theoretical plates (Efficiency)	Not less Than 2000	4668	3868	6238
Tailing factor (T)	Not More Than 2	1.07	0.92	1.33

* Mean of six injections

In order to test the applicability of the method developed to a commercial formulation, 'NERVUP PG' was chromatographed and it is represented in **Figure 9**. The sample peaks were identified by comparing the relative retention times with the standard drugs mixture, **Figure 8**. System suitability parameters were ideal for the chromatographed sample. Integration of separated peak area was done and each drug was determined by using the peak area-concentration relationship obtained in the standardization step. The protocol affords reproducible quantification of the three drugs with error less than 10%, which is the standard level in any pharmaceutical quality control.

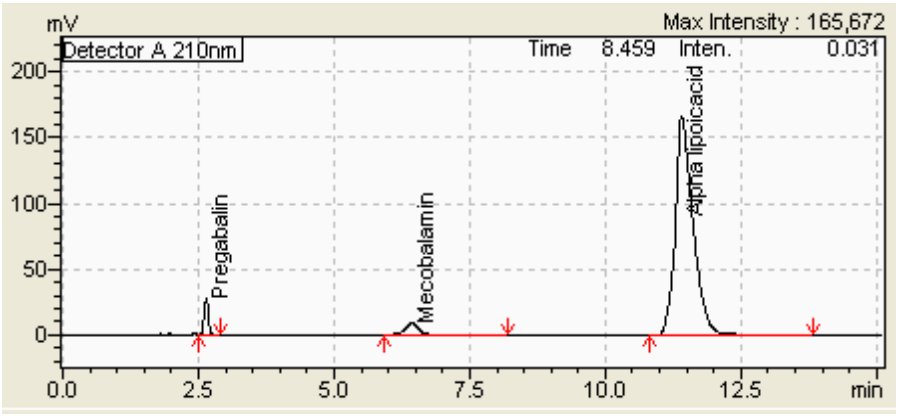

Fig. 9: Typical chromatogram for the sample (capsule).

Method validation

Validation of the analytical method is the process that establishes by laboratory studies in which the performance characteristics of the method meet the requirements for the intended analytical application. RP-HPLC method developed was validated according to International Conference on Harmonization (ICH) guidelines [24] for validation of analytical procedures. The method was validated in terms of parameters like system suitability, selectivity, linearity, accuracy, precision, ruggedness, robustness, limit of detection (LOD) and limit of quantitiation (LOQ).

Specificity

Figures 7-9 for blank, mixture of standard drug solution and sample reveal that the peaks observed in mixture of standard solution and sample solution are only because of the drugs as blank has no peaks at the retention times of Pregabalin, Mecobalamin and Alpha Lipoc acid. Accordingly, the method developed is said to be specific.

Precision

System precision

Six replicate injections of the mixture of standard solution at working concentration showed % RSD (Relative Standard Deviation) less than 2 concerning peak areas for the three drugs, which indicates the acceptable reproducibility and thereby the precision of the system. System precision results are tabulated in **Table 2**.

Method precision

Method precision was determined by performing assay of sample under the tests of repeatability (Intra day precision) and Intermediate precision (Inter day precision) during 3 days at working concentration.

Repeatability (Intra day precision)

Six consecutive injections of the sample at working concentration showed % RSD less than 2 concerning %assay and peak areas for all the three drugs which indicate the method developed is method precise by the test of repeatability and hence the method should give consistently reproducible results (**Table 3)**.

Ruggedness (Intermediate Precision / Inter day precision/)

Six consecutive injections of the sample solution at working concentration on three consecutive days by three different analysts, showed % RSD 2 for % assay for all the drugs, which indicate the method developed is inter day precise / rugged (**Table 4)**.

Linearity

Standard solutions of Pregabalin, Mecobalamin and Alpha lipoic acid of different concentrations level (50%, 75%, 100%, 125%, 150%, 175% and 200%) were prepared in triplicate. Calibration curves were constructed by plotting the % concentration levels of drugs versus corresponding mean peak area. The results show an excellent correlation exists between mean peak area and % concentration levels of drugs within the concentration range of Pregabalin(187.5-750µg/ml), Mecobalamin(1.87-7.5µg/ml), Alpha lipoic acid (250-1000 µg/ml) and the results are given in **Tables 5-8** and **Figures 10-12.** The correlation coefficients of Pregabalin, Mecobalamin and Alpha lipoic acid are greater than 0.999, which meet the method validation acceptance criteria and hence the method developed is said to be linear in the range of Pregabalin(187.5-750µg/ml), Mecobalamin(1.87-7.5µg/ml), Alpha lipoic acid(250-1000µg/ml).

Table 2: System precision results

Injection number (n)	Pregabalin		Mecobalamin		Alpha lipoic acid	
	Rt	Peak area	Rt	Peak area	Rt	Peak area
1	2.64	`158916	6.41	189218	11.34	4275032
2	2.64	159955	6.39	186236	11.35	4186045
3	2.63	158232	6.41	191228	11.35	4154238
4	2.64	157298	6.39	186427	11.34	4185042
5	2.63	157535	6.4	189228	11.36	4178142
6	2.63	156130	6.42	190229	11.35	4117312
Average		**157830**		**188761**		**4181874**
S.D.		**1408.65**		**2024.41**		**24537.9**
% R.S.D.		**0.89**		**1.07**		**0.58**

Table 3: Intra day precision results

n	PREGABALIN	MECOBALAMIN	ALPHA LIPOIC ACID
	% Assay	% Assay	% Assay
1	100.31	100.52	101.1
2	100.96	98.94	100.16
3	99.88	101.59	99.4
4	99.29	99.04	100.14
5	99.44	100.53	99.97
6	98.55	101.06	98.55
Average	**99.73**	**100.28**	**99.88**
S.D.	**0.84**	**1.07**	**0.85**
% R.S.D.	**0.84**	**1.07**	**0.85**

Table 4: Inter day precision results

n	% Assay PREGABALIN			% Assay MECOBALAMIN			% Assay ALPHA LIPOIC ACID		
	Day 1	Day 2	Day 3	Day 1	Day2	Day 3	Day 1	Day2	Day 3
1	100.31	99.15	99.18	100.5	99.20	98.85	101.1	99.49	99.12
2	100.96	99.82	99.35	99.94	99.03	99.06	100.16	99.15	99.32
3	99.88	99.01	99.02	101.5	100	99.95	99.4	99.73	100.6
4	99.29	101.3	99.75	99.04	100.5	100..6	100.14	99.84	100.8
5	99.44	99.50	101	100.5	99.8	101.5	99.97	99.50	99.29
6	98.55	99.44	99.89	101.0	99.3	100.6	98.55	99.36	99.74
Average	**99.73**	**99.7**	**99.69**	**100.2**	**99.64**	**99.99**	**99.88**	**99.51**	**99.82**
S.D.	**0.84**	**0.83**	**0.72**	**1.07**	**0.56**	**1.1**	**0.85**	**0.25**	**0.72**
% R.S.D.	**0.84**	**0.83**	**0.72**	**1.07**	**0.56**	**1.1**	**0.85**	**0.25**	**0.72**

Table 5: Calibration data for Pregabalin

Actual Concentration (µg/ml)	% Concentration Level	Peak Area
187.5	50	79211
281.25	75	120818
375	100	158421
468.75	125	198027
562.5	150	241636
656.25	175	278796
750	200	318625

Table 6: Calibration data for Mecobalamin

Actual Concentration (µg/ml)	% Concentration Level	Peak Area
1.87	50	94013
2.81	75	141320
3.75	100	188226
4.68	125	235033
5.62	150	282020
6.56	175	329327
7.5	200	376264

Table 7: Calibration data for Alpha lipoic acid

Actual Concentration (µg/ml)	% Concentration Level	Peak Area
250	50	2089514
375	75	3133572
500	100	4179027
625	125	5223806
750	150	6268641
875	175	7313210
1000	200	8358154

Table 8: Linearity of the chromatography system

Drugs	Linearity range (µg/ml)	R^2	Slope	Intercept
Pregabalin	187.5-750	0.999	1596.304	-176.036
Mecobalamin	1.87-7.5	0.999	1880.801	71.67857
Alpha lipoic acid	250-1000	0.999	41792.59	-369.786

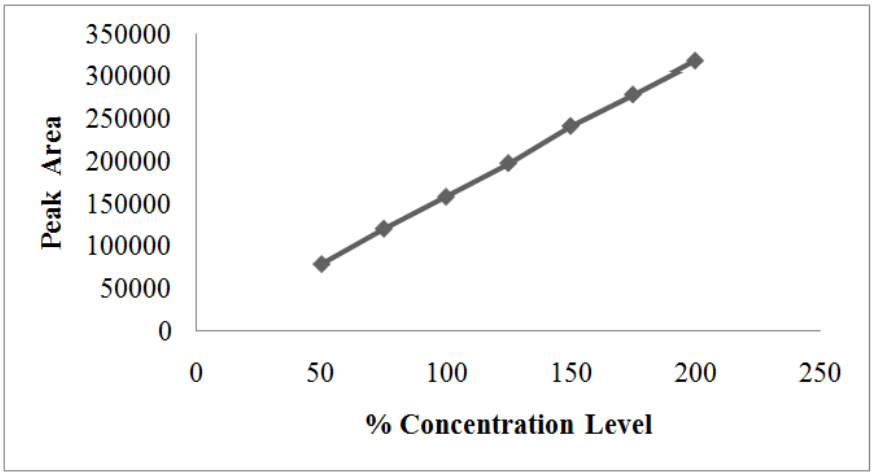

Fig.10: Calibration curve for Pregabalin.

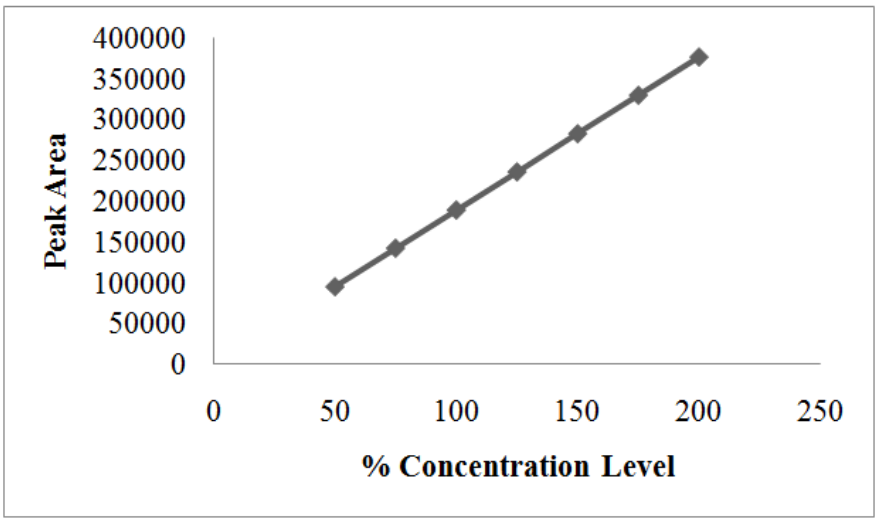

Fig.11: Calibration curve for Mecobalamin.

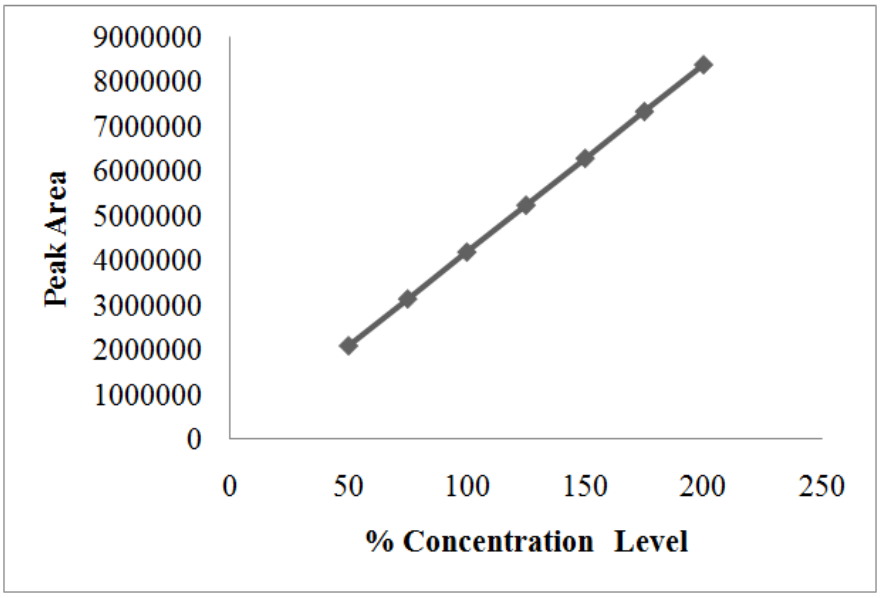

Fig.12: Calibration curve for Alpha Lipoic acid.

Table 9: Results of Accuracy studies for Pregabalin

Concentration level (%)	Amount added (µg/ml)	*Amount recovered (µg/ml)	*% Recovery
50	187.5	188.76	100.67
100	375	373.3	99.54
150	562.5	563.43	100.16

*Mean of three replicates

Table 10: Results of Accuracy studies for Alpha Lipoic acid

Concentration level (%)	Amount added (µg/ml)	*Amount recovered (µg/ml)	*% Recovery
50	250	249.36	99.74
100	500	501.1	100.22
150	750	749.25	99.9

*Mean of three replicates

Table 11: Results of Accuracy studies for Mecobalamin

Concentration level (%)	Amount added (µg/ml)	*Amount recovered (µg/ml)	*% Recovery
50	1.875	1.870	99.73
100	3.75	3.76	100.26
150	5.625	5.65	100.44

*Mean of three replicates

Accuracy

Accuracy was determined by means of recovery experiments, by the addition of active drug to preanalyzed sample at different spiked levels (50-150%). At each level, three determinations were performed and results obtained. The accuracy was calculated from the test results as the percentage of the analyte recovered by the assay. The amounts recovered, values of percent mean recovery were calculated as shown in **Tables 9-11.** The accepted limits of recovery are 98%-102% and all observed data are within the required range that indicates good recovery values and hence the accuracy of the method developed.

Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. It is concluded that the method is robust as it is found that the % RSD is less than 2 concerning % assay despite deliberate variations done concerning flow rate (± 0.2), pH (± 0.2) and % organic phase (± 5%).

Sensitivity

The sensitivity of measurement of Pregabalin, Mecobalamin and Alpha lipoic acid by use of the proposed method was estimated in terms of LOQ) and LOD.The limit of detection (LOD) was obtained as 3µg/ml for Pregabalin, 800ng/ml for Mecobalamin and 0.5µg/ml for Alpha lipoic acid. The limit of quantitation (LOQ) was obtained as 6µg/ml for Pregabalin, 2µg/ml for Mecobalamin and 1.5µg/ml for Alpha lipoic acid.

CONCLUSION

A reverse phase HPLC isocratic method developed has been validated as per ICH guidelines in terms of specificity, accuracy, precision, linearity, ruggedness, robustness, limit of detection and limit of quantitiation, for the simultaneous quantitative estimation of Pregabalin, Mecobalamin and Alpha lipoic acid in capsules. A good linear relationship was observed for the three drugs between concentration ranges of (187.5-750µg/ml), (1.87-7.5 µg/ml) and (250-1000µg/ml). The correlation coefficients were greater than 0.999 for three drugs. The inter day and intraday precision results were good enough to say that the method developed is precise and reproducible. Accuracy studies revealed that mean recoveries after spiking experiments were between 98 and 102%, an indicative of accurate method. Accordingly it can be concluded that the developed reverse phase HPLC isocratic method is accurate, precise, linear, rugged and robust. Accordingly, the method can be used for the routine analysis of Pregabalin, Mecobalamin and Alpha lipoic acid in capsules.

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