"Development and Validation of an RP-HPLC Method for the Simultaneous Estimation of Nortriptyline, Pregabalin, and Methylcobalamin in Pharmaceutical Dosage Forms"

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Abstract:

This study shows a validated RP-HPLC method. It allows for the simultaneous measurement of Nortriptyline, Pregabalin, and Methylcobalamin. These drugs are commonly used to treat neuropathic pain. The method used a Zodiac-C8 column (5µm, 150 × 4.6 mm ID). The mobile phase included 10mM KH_2PO_4 (solvent A) and acetonitrile-methanol (80:20, v/v) (solvent B). The flow rate was set at 1.0 mL/min. The detection wavelength was set at 230 nm, and separation was achieved within 15 minutes. Method validation followed ICH guidelines, evaluating linearity, precision, accuracy, robustness, and system suitability. The method showed excellent linearity ($R^2 \approx 0.999$) across the tested concentration range, with %RSD < 2% for intra- and interday precision. Accuracy studies confirmed drug recoveries within 100–105%, ensuring high reliability. Testing showed that small changes in flow rate, organic modifier concentration, and wavelength led to only minor variations.

The C8 column showed better separation and retention than traditional C18 methods. It successfully tackled the challenges in analyzing various drugs at the same time. The RP-HPLC method is very accurate, precise, and robust. This makes it a great tool for checking pharmaceutical quality. It works well for routine drug analysis in both commercial formulations and biological samples.

Keywords: *RP-HPLC, Nortriptyline, Pregabalin, Methylcobalamin, Simultaneous Quantification*.

1. INTRODUCTION

Chromatographic techniques are essential in pharmaceutical analysis. They are vital for drug development, quality control, and regulatory compliance. Reverse Phase Chromatography (RPC) and Mixed-Mode Chromatography (MMC) help estimate multiple pharmaceutical compounds simultaneously. These techniques work well when using combination drug therapies. This is true for treating hypertension and neuropathic disorders. As drug formulations become more complex, it’s crucial to choose the right chromatographic technique. This choice ensures drug effectiveness and keeps patients safe (Snyder et al., 2016).

Hypertension and neuropathy are common chronic conditions. They often need long-term treatment with several medications. Antihypertensive drugs help manage high blood pressure. They include calcium channel blockers, beta-blockers, and angiotensin receptor blockers. These medications also prevent cardiovascular complications (Kirkland et al., 2018). Neuropathic pain comes from nerve damage. This can happen from diabetes, chemotherapy, or injury. To treat it, doctors use drugs like gabapentinoids, tricyclic antidepressants, and SNRIs (Baron et al., 2017). Patients with hypertension and neuropathy often take several medications at once. It’s important to develop ways that measure these drugs in complex mixtures and biological samples (Shah et al., 2020).

Reverse Phase Chromatography (RPC) is a popular technique in liquid chromatography. It is widely used in high-performance liquid chromatography (HPLC). It is based on the principle of hydrophobic interactions between the stationary phase and the analyte molecules. In RPC, the stationary phase uses non-polar materials like C18 or C8 bonded silica. The mobile phase has polar solvents such as water, methanol, or acetonitrile. These solvents often include buffer solutions to adjust the pH. In RPC, compounds separate based on hydrophobicity. More hydrophobic compounds stay on the stationary phase longer (Dolan, 2018). This method provides high resolution, reproducibility, and sensitivity. So, it's perfect for analyzing pharmaceutical compounds, like hydrophobic and moderately polar drugs. RPC estimates different antihypertensive and neuropathic drugs well. This is because these drugs have different chemical structures (Kromidas, 2017).

RPC has benefits, but it has limits. This is especially true for highly polar or ionizable compounds. RPC mainly depends on hydrophobic interactions. This means that hydrophilic or charged molecules might not stick well. As a result, they can show low retention or resolution. This poses challenges when developing methods. This is where Mixed-Mode Chromatography (MMC) presents a promising alternative. MMC uses different retention methods in one chromatographic system. These methods include hydrophobic, ion-exchange, hydrogen bonding, and π–π interactions (Zhang & Henion, 2019). MMC stands out from traditional RPC. While RPC only uses hydrophobicity for separation, MMC offers better selectivity and flexibility. MMC is ideal for complex pharmaceutical mixtures. It works well with both hydrophobic and hydrophilic compounds.

A key advantage of MMC is that it enhances the separation of various drugs. This includes drugs that are acidic, basic, or neutral. This is especially useful for estimating antihypertensive and neuropathic drugs together. These drugs often have a wide range of physicochemical properties. Beta-blockers, like propranolol and metoprolol, are fairly hydrophilic. In contrast, calcium channel blockers, such as amlodipine, are more hydrophobic. Neuropathic drugs like gabapentin and pregabalin are very polar. Tricyclic antidepressants like amitriptyline have strong hydrophobic interactions (Berthod et al., 2019). MMC can adjust mobile phase conditions and stationary phase interactions. This flexibility makes it a stronger and more versatile analytical method than RPC.

We need a comparative study of RPC and MMC. This will help us find the best method for estimating drugs at the same time. Consider several factors: retention time, resolution, peak symmetry, sensitivity, reproducibility, and robustness. The mobile phase, column chemistry, and detection methods—such as UV, fluorescence, or mass spectrometry—impact the effectiveness of each chromatographic method (Liu et al., 2020). RPC is well-known for its efficiency with many pharmaceutical compounds. However, MMC offers better selectivity for complex mixtures. This makes MMC a promising option for tough analytical situations (Rizwan et al., 2021).

This study compares the strengths and weaknesses of two chromatographic methods. It focuses on their ability to estimate certain antihypertensive and neuropathic drugs at the same time. This study looks at key performance parameters. It aims to find the best method for pharmaceutical quality control, regulatory compliance, and routine lab use. Understanding the differences between RPC and MMC helps researchers and analysts enhance chromatographic conditions. It also supports the growth of analytical methods in pharmaceutical sciences. This study will help measure drugs accurately. It will also improve treatment effectiveness and boost patient safety in clinical practice (Patel et al., 2022).

1. **Nortriptyline**

Nortriptyline is a tricyclic antidepressant primarily used to treat depression. It works by increasing the levels of norepinephrine and serotonin in the brain, which helps stabilize mood and reduce symptoms of depression (MedlinePlus, n.d.). Nortriptyline is often given off-label for neuropathic pain and to prevent migraines (NCBI, 2021).

Common side effects are:

- Drowsiness
- Dizziness
- Dry mouth
- Constipation
- Weight gain
- Increased heart rate (NHS, 2023)

Serious issues, like arrhythmias or seizures, can occur. This is especially true for those with heart problems (NCBI, 2021). Nortriptyline should not be taken with monoamine oxidase inhibitors (MAOIs) due to the risk of serotonin syndrome (MedlinePlus, n.d.).

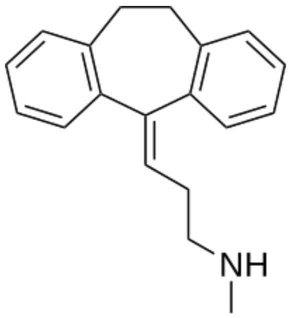

Figure 1 Chemical Structure of Nortriptylene

2. Methylcobalamin

Methylcobalamin, an active form of vitamin B12, plays an essential role in nerve function, red blood cell formation, and DNA synthesis (Mayo Clinic, n.d.). It is often prescribed for vitamin B12 deficiency, peripheral neuropathy, and megaloblastic anemia (Drugs.com, 2023). Methylcobalamin helps turn homocysteine into methionine. This process is vital for brain health and keeps the myelin sheath intact (PubMed Central [PMC], 2014). This vitamin is usually well-tolerated. Mild side effects like diarrhea, itching, or rash may happen, but they are rare (Drugs.com, 2023). However, individuals with Leber’s disease, a hereditary optic neuropathy, should avoid its use as it may worsen symptoms (Cigna, n.d.). Methylcobalamin can also interact with some medications. For example, it can reduce the effectiveness of chloramphenicol (Drugs.com, 2023).

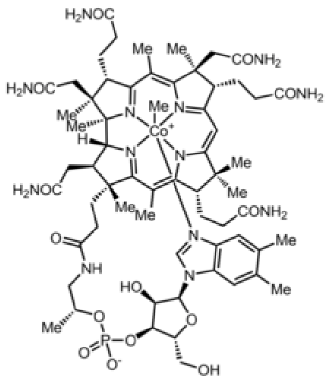

Figure 2 Chemical Structure of Methylcobalamin

3. Pregabalin

Pregabalin is an anticonvulsant and pain reliever. It treats conditions such as diabetic neuropathy, post-herpetic neuralgia, fibromyalgia, and generalized anxiety disorder (Drugs.com, 2023). It works by binding to calcium channels in the central nervous system, reducing the excessive release of excitatory neurotransmitters such as glutamate, norepinephrine, and substance P (MedlinePlus, n.d.).

Pregabalin can cause side effects like:

- Dizziness
- Drowsiness
- Blurred vision
- Dry mouth
- Weight gain
- Fluid retention (Drugs.com, 2023).

Patients might experience mood changes, suicidal thoughts, or withdrawal symptoms if they suddenly stop their medication (NCBI, 2018). Pregabalin can be abused and lead to dependence. So, it's important to use it carefully, especially in people who have a history of substance abuse (Drugs.com, 2023). Patients with kidney impairment may require dosage adjustments to prevent toxicity (Cleveland Clinic, n.d.).

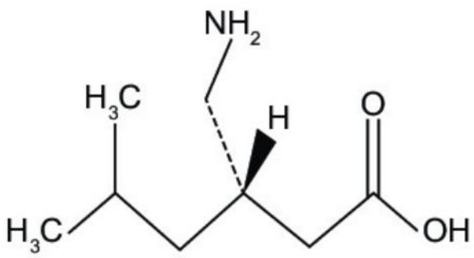

Figure 3 Chemical Structure of Pregabalin

2. MATERIALS AND METHODS

2.1. Instrumentation

We used the Shimadzu SCL-10AVP high performance liquid chromatography (HPLC) system. It has a binary pump (LC-10ATVP), a UV detector (SPD-10AVP), and a manual injector with a 20µl loop capacity (P/N 77251). The LC-Solution software was used to interpret the HPLC reports. Zodiac-C18 (5µm; 150 x 4.6 mm ID.) column purchased from Zodiac life sciences was used throughout the analysis. Digital weighing balance (ME-204) by Mettler-Toledo (USA) and Labman® ultra-sonicator from UltraChrom Ltd, India. Digital pH meter from Mettler-Toledo was purchased from (Mumbai-India). 50 µ micro-syringe was purchased from Hamilton USA. 0.20µ and 0.45µ nylon membrane filters were purchased from Phenomenex® Mumbai, India.

2.2. Reagents and reference samples

The reference standard includes Methyl-cobolamin, Pregabalin, and Nortriptyline. These were purchased from Yarrow Chem Ltd in Mumbai, India. Acetonitrile (ACN), methanol (MeOH), deionised water, and potassium dihydrogen phosphate were HPLC grade. They were purchased from Merck Ltd. (Mumbai-India). 0.20µm nylon membrane filters were used for sample filtrations and were purchased from UltraChrom Innovatives Pvt. Ltd. (India). All other chemicals and reagents were used of HPLC grade.

2.3 Selection of solvent and wavelength

Methyl-cobolamin, Pregabalin, and Nortriptyline dissolve in methanol. They are also partly soluble in water and acetonitrile. So, a standard stock solution of the chosen neuropathic pain inhibitors was made using acetonitrile, methanol, and water in a 40:40:20% mix.

2.4 Preparation of standard solution

Weigh out 7 mg of each standard: Methyl-cobalamin, Pregabalin, and Nortriptyline. Then, dissolve them in 7 ml of a solution made from acetonitrile, methanol, and water (3:3:1, v/v). This creates a 1000 ppm (1000 µg/ml) solution. We sonicated it for 2 to 7 minutes to ensure full dissimilation. Then, we made serial dilutions to reach the final concentrations: methylcobalamin at 10 ppm, Pregabalin at 500 ppm, and Nortriptyline at 70 ppm. This was done for repeatability, precision, and robustness studies. (Potluri et al., 2017; Ponnekanti et al., 2021).

2.5 Chromatographic conditions

We injected 20µl of a fresh mixture into the Zodiac-C8 column. This mixture contained methylcobalamin (10 ppm), Pregabalin (500 ppm), and Nortriptyline (70 ppm). The column specifications are 5µm with a size of 150 x 4.6 mm ID.) column and eluted using the mobile phase as solvent A; 10mM $KH2PO4$ and solvent B; acetonitrile-methanol (80:20, v/v) at 1.0 ml/mins flow rate for 15 mins. Separation was carried out at 31˚C and monitored at 230 nm wavelength.

2.6 System suitability studies

We prepared a fresh mixture of stock solution with the following: methylcobalamin (10 ppm), Pregabalin (500 ppm), and Nortriptyline (70 ppm). We injected this mixture six times to check the consistency of results. The relative standard deviation (RSD) should always be less than 2%.

Other system suitability parameters were also calculated. These include retention time (tR), capacity factor (k'), theoretical plates (N), tailing factor/peak asymmetry (As), and separation factor (α).

2.7. Sample preparation for drug accuracy studies

Exactly 5 tablets of Cobivital NTM Tablet manufactured by Leeford pharmaceuticals Ltd. Pregabalin (75 mg), Methylcobalamin (1.5 mg), and Nortriptyline (10 mg) were weighed. Then, we calculated the average weight. They were mixed and crushed to fine powder into the mortar and pestle. Weigh 10 mg of isavuconazole powder. Then dissolve it in 10 ml of a mix of acetonitrile, methanol, and water (4:4:2, v/v) to create a 1000 ppm solution. It was ultrasonicated for 5-10 mins and filtered through 0.45µ nylon filter. Also, we made serial dilutions to get the final concentration matching the tablet dosage. The solution was then sonicated and analysed as per the chromatographic condition mentioned in section 5.5.

2.8. Sample preparation for Linearity/Calibration studies

A fresh stock solution of 1000 ppm (1000 µg/ml) was prepared for each standard: Pregabalin, Methylcobalamin, and Nortriptyline.

Also, we made a mixture with five serial dilutions. The concentrations were:

- Pregabalin: 7.8–250 µg/ml
- Methylcobalamin: 0.3–10 µg/ml
- Nortriptyline: 1.09–35 µg/ml

They were sonicated and analysed as per the chromatographic condition in section 5.x. The calibration curve was plotted by calculating the peak area for known concentrations. This helped us find the regression equation, the regression coefficient (R2), the limit of quantification (LOQ), and the limit of detection (LOD).

2.9. Precision studies of the proposed method

We prepared a fresh mixture of the stock solution. It contained methylcobalamin (10 ppm), Pregabalin (500 ppm), and Nortriptyline (70 ppm). We analyzed this mixture three times in one day for intraday precision. Then, we tested it over three consecutive days for intermediate precision. Also, we calculated the mean, standard deviation, and relative standard deviation (RSD). The RSD should be less than 2%, according to ICH guidelines.

2.10 Robustness for the chromatographic method

We changed the mobile phase flow rate by 1.00 ±1 decimal. It went from 1 mL/min to 1.1 mL/min and then to 0.9 mL/min. We evaluated how flow rate affects the separation of Pregabalin, Methylcobalamin, and Nortriptyline. Also, we changed the amount of organic modifier in solvent B to 28% and 32% by adjusting it ±2% from 30%. This helped us study the effects on retention time (tR), capacity factor (k'), and theoretical plates (N). We monitored the effect of wavelength by varying it from 230±2 nm to 228 and 232 nm. We then tested and evaluated the differences in retention time (tR), capacity factor (k'), resolution (Rs), and theoretical plates (N).

3. RESULTS AND DISCUSSION

No one has tried to measure Methylcobalamin, Pregabalin, and Nortriptyline at the same time. Most studies only look at pairs of these pain relievers. This study offers a new way to analyze them at the same time.

We used a polar stationary phase-packed C8 column instead of traditional C18 columns. This choice improved both separation efficiency and retention features. This approach enhances peak resolution and selectivity for the analytes.

The retention/capacity factor (k') is important in chromatographic analysis. However, it hasn't been reported in earlier studies. Many current methods show that the first eluting analyte appears at or near the void volume (t_0). This does not comply with ICH guidelines. Our method makes sure the first separated compound has a retention factor (k') over 0.5. This meets regulatory standards and boosts method reliability.

The method follows ICH (International Conference on Harmonization) guidelines for validation. This ensures accuracy and precision when measuring Methylcobalamin, Pregabalin, and Nortriptyline together. UV spectral analysis of methyl-cobalamin

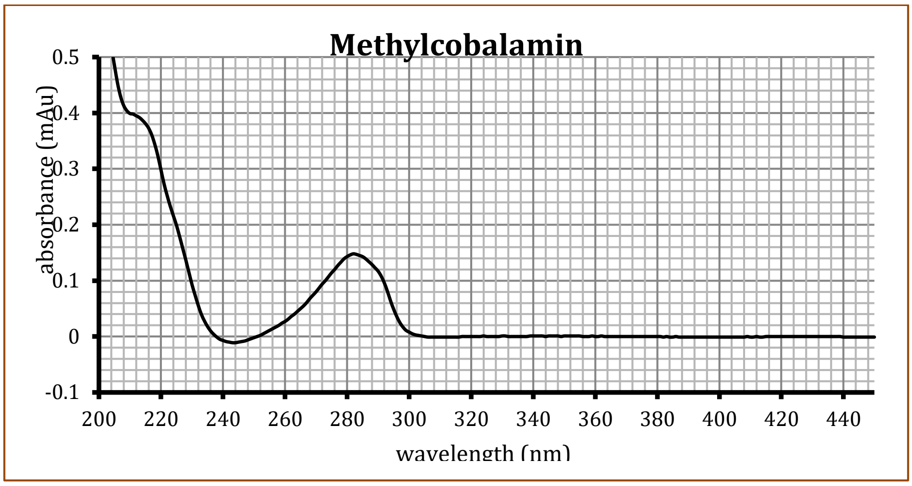

Figure 4; UV spectral analysis of Methyl-cobalamin

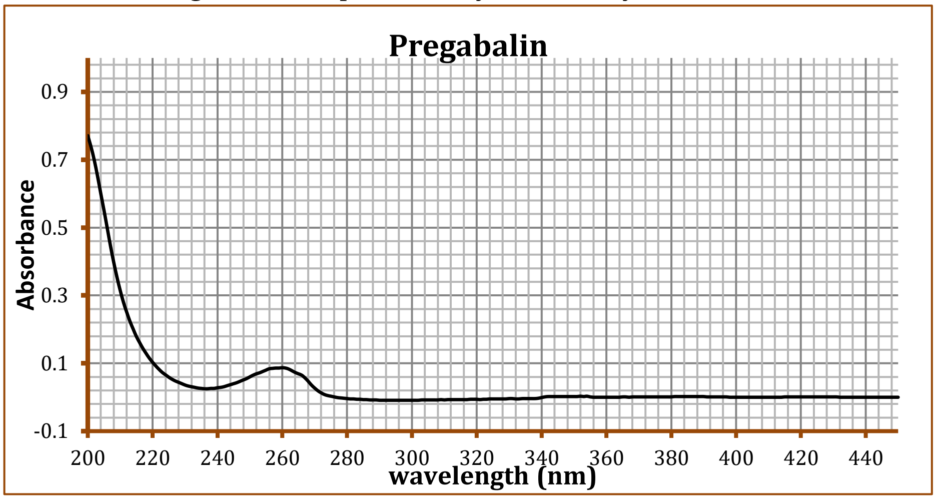

Figure 5; UV spectral analysis of Pregabalin

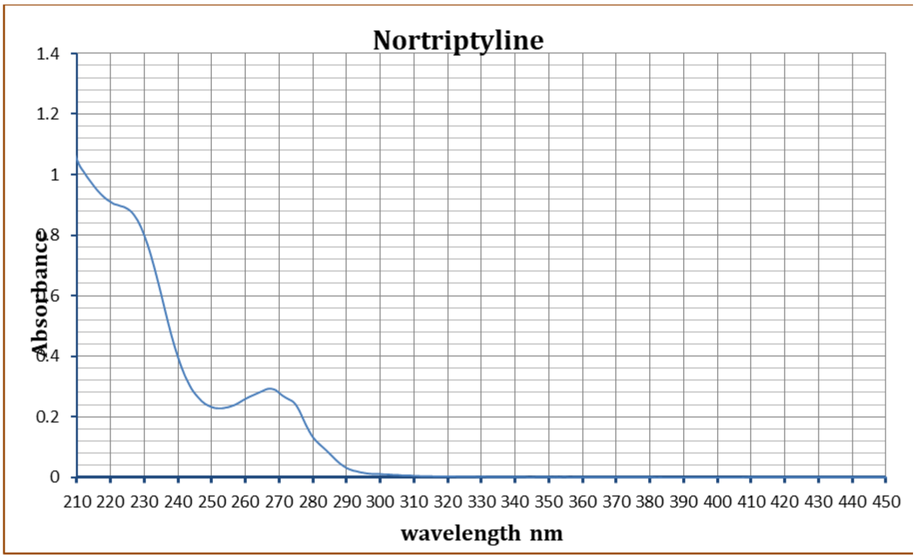

Figure 6; UV spectral analysis of Nortriptyline

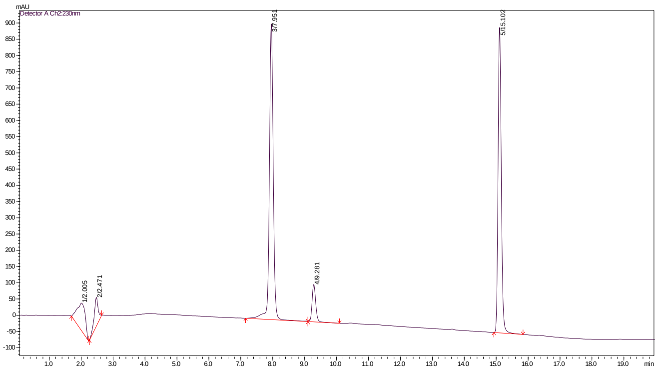

Figure 7; trial 1 of estimation of pregabalin, Nortriptyline and methylcobalamin by RP-HPLC

Table 1; Trial 1st for simultaneous estimation of neuropathic pain inhibitor

Peak#	Ret. Time	Area	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
2	2.471	779265	4.4367	1864.288	1.291	0.233	0.93	0
Nortriptyline	7.951	7720889	43.9581	20957.62	24.427	2.966	1.085	12.748
Methyl-cobalamin	9.281	916606	5.2186	30090	6.135	3.629	1.2	1.224
Pregabalin	15.102	6645634	37.8362	96694.96	28.515	6.532	1.196	1.8

Analytes: nortriptyline, pregabalin and methylcobalamin

Column: Zodiac C8 (5µ, 150 X 4.6mm. ID.)

Mobile Phase: solvent A, 0.1% TFA; solvent B, acetonitrile-methanol (70:30, v/v)

Flow rate: 1ml/min

Elution mode: gradient elution mode

Elution program: 0-3 mins, 30% B; 3-15 mins, 80% B

Wavelength selected: 230 nm

Temperature: Room temperature

Discussion; baseline was not constant.

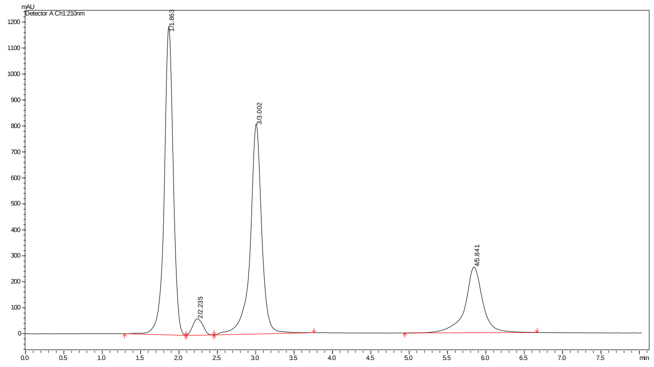

Figure 8; trial 2 of estimation of pregabalin, nortriptyline and methylcobalamin by HILIC

Table 2; Trial 2nd for simultaneous estimation of neuropathic pain inhibitor

Peak#	Ret. Time	Area	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.863	9666339	42.4089	1200.761	--	0	0.958	0
Pregabalin	2.235	584386	2.5639	1240.89	1.586	0.2	1.16	0
Nortriptyline	3.002	8478643	37.1982	2260.452	3.032	0.612	0.893	3.065
Methyl-cobalamin	5.841	4063812	17.8291	4028.338	9.146	2.136	0.909	3.491

Analytes: methylcobalamin, pregabalin and nortriptyline

Column: Acclaimed mix-mode HILIC-1 (5μ, 150 X 4.6mm. ID.)

Mobile Phase: solvent A, 15 mM AA; solvent B, acetonitrile

Flow rate: 1ml/min

Elution mode: gradient elution mode

Elution program: 0-3 mins, 30% B; 3-15 mins, 80% B

Wavelength selected: 230 nm

Temperature: Room temperature

Discussion; methyl-cobolamin was eluted with the void volume and the retention factor was quite less.

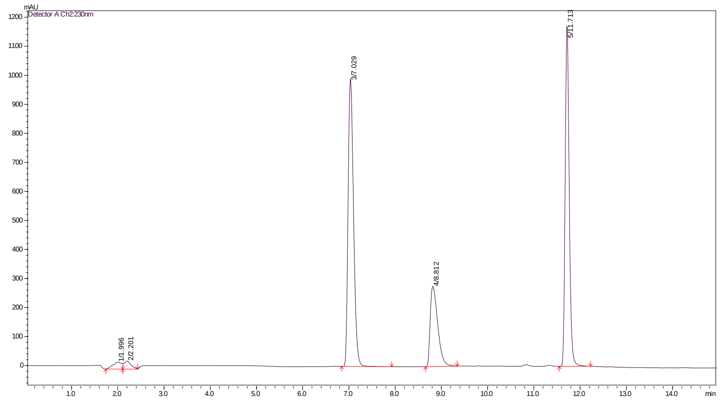

Figure 9; method development; estimation of pregabalin, nortriptyline and methylcobalamin

Table 3; method development for simultaneous estimation of neuropathic pain inhibitor

Peak#	Ret. Time	Area	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
2	2.201	282521	1.4906	530.906	0.426	0.103	--	0
Nortriptyline	7.029	8088647	42.6773	15772.05	15.936	2.522	1.341	24.539
Methyl-cobalamin	8.812	3121593	16.4702	13556.69	6.769	3.415	1.505	1.354
Pregabalin	11.713	7140519	37.6748	74774.37	12.24	4.869	1.284	1.426

Analytes: nortriptyline, pregabalin and methylcobalamin

Column: Zodiac C8 (5μ, 150 X 4.6mm. ID.)

Mobile Phase: solvent A, 10 mM KH2PO4; solvent B, acetonitrile-methanol (90:10,v/v)

Flow rate: 1ml/min

Elution mode: gradient elution mode

Elution program: 0-3 mins, 30% B; 3-15 mins, 80% B

Wavelength selected: 230 nm

Temperature: Room temperature

2. Method validation

The method was validated according to ICH guidelines.

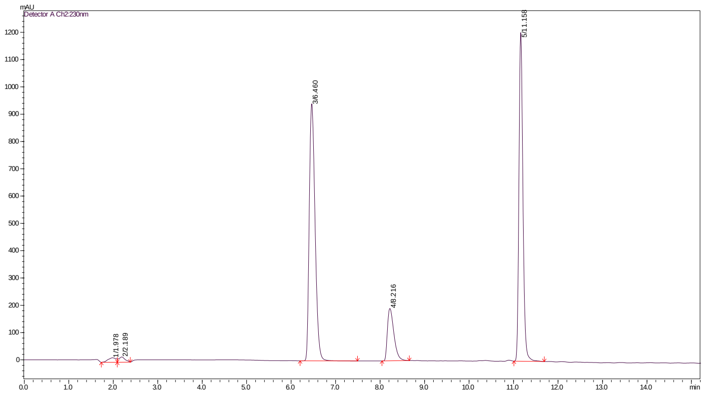

Figure 10; validation study; estimation of pregabalin, nortriptyline and methylcobalamin

Table 4; validation study; for simultaneous estimation of neuropathic pain inhibitor

Peak#	Ret. Time	Area	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
2	2.189	187000	1.0154	723.073	0.507	0.107	--	0
Nortriptyline	6.46	8524260	46.2858	11010.01	14.934	2.266	1.359	21.228
Methyl-cobalamin	8.216	1946828	10.5711	14660.41	6.787	3.154	1.657	1.392
Pregabalin	11.158	7551673	41.0047	65960.94	13.215	4.641	1.284	1.472

Analytes: nortriptyline, pregabalin and methylcobalamin

Column: Zodiac C8 (5µ, 150 X 4.6mm. ID.)

Mobile Phase: solvent A, 10 mM KH2PO4; solvent B, acetonitrile-methanol (90:10, v/v)

Flow rate: 1ml/min

Elution mode: gradient elution mode

Elution program: 0-3 mins, 30% B; 3-15 mins, 80% B

Wavelength selected: 230 nm

Temperature: Room temperature

2.1. Repeatability study

We followed the steps in section 5.3. We tested a mixture of nortriptyline, pregabalin, and methylcobalamin with six injections in one day. The % RSD was calculated and found it is less than 2% for nortriptyline, pregabalin and methylcobalamin.

Table 5; Repeatability data of selected neuropathic pain inhibitors

S. No.	Drug Name; **Pregabalin**	Drug Name: **Methylcobalamin**	Drug name: **Nortriptyline**
	Peak Area; Conc. 500 ppm	Peak Area; Conc. 10 ppm	Peak Area; Conc. 70 ppm
1	8474339	1779584	7655260
2	8524260	1746828	7551673
3	8450294	1772796	7726354
4	8495044	1737841	7621058
5	8288511	1750479	7593929
6	8326197	1762220	7492049
Mean	**8426440.833**	**1758291.333**	**7606720.5**
STD. DEV.	**96137.0447**	**16061.47021**	**81507.32965**
RSD (%)	**1.14**	**0.91**	**1.07**

2.3 System suitability studies

The RP-HPLC method for measuring Nortriptyline, Pregabalin, and Methylcobalamin was validated following ICH guidelines. System suitability parameters, including repeatability, linearity, precision, and robustness, met acceptance criteria. The tailing factor (T) was less than 2, which ensured peak symmetry. The separation factor (α) and resolution (Rs) both met and surpassed the minimum ICH requirements. The method showed great reproducibility. Key system suitability parameters stayed within acceptable limits across six injections. These included theoretical plates (N), capacity factor (k’), resolution (R), separation factor (α), and tailing factor (T) (see Table 6.1). 6; System suitability of nortriptyline, pregabalin and methylcobalamin

2.4. Precision studies for nortriptyline, pregabalin and methylcobalamin

The HPLC method demonstrated high precision, with %RSD <2% across multiple samplings of the same drug mixture. Intermediate precision studies confirmed consistency over the tested range for Nortriptyline, Pregabalin, and Methylcobalamin. The peak area correlated well with

concentration, ensuring minimal variation and method reliability (Table 2).

2.5. Intraday precision

We analyzed the homogeneous mixture of Nortriptyline, Pregabalin, and Methylcobalamin. We followed the procedure from Section 5.3. The tests were done in triplicate over three days. This helped us check interday and intermediate precision. The %RSD remained <2% for all analytes, confirming high precision (Tables 6.4.1–6.4.2).

Table 7 Intraday precision data of nortriptyline

Drug Name: nortriptyline				
S. No.	Concentration (ppm)	Area	Std. Deviation	%RSD
1	70 PPM	8474339	37729.82335	0.44
	70 PPM	8524260		
	70 PPM	8450294		
2	70 PPM	8495044	109988.9571	1.31
	70 PPM	8288511		
	70 PPM	8326197		
3	70 PPM	8544530	49524.65625	0.58
	70 PPM	8451020		
	70 PPM	8526059		
	Range of % RSD			**0.44-1.31**

Table 8; Intraday precision data of pregabalin

Drug Name: pregabalin				
S. No.	Concentration (ppm)	Area	Std. Deviation	%RSD
1	500 PPM	1779584	17288.57766	0.98
	500 PPM	1746828		
	500 PPM	1772796		
2	500 PPM	1737841	12192.25004	0.70
	500 PPM	1750479		
	500 PPM	1762220		
3	500 PPM	1617772	7414.895167	0.46
	500 PPM	1603526		
	500 PPM	1614217		
	Range of % RSD			**0.46-0.98**

Table 9; Intraday precision data of methylcobalamin

Drug Name: methylcobalamin				
S. No.	Concentration (ppm)	Area	Std. Deviation	%RSD
1	10 ppm	7655260	87842.73368	1.15
	10 ppm	7551673		
	10 ppm	7726354		
2	10 ppm	7621058	68018.18277	0.90
	10 ppm	7593929		
	10 ppm	7492049		
3	10 ppm	7561777	57533.61354	0.76
	10 ppm	7560352		
	10 ppm	7461421		
	Range of % RSD			**0.76-1.15**

2.5. Interday (intermediate) precision

The homogeneous mixture of Nortriptyline, Pregabalin, and Methylcobalamin was analyzed in triplicate. This testing took place over three days to check interday and intermediate precision. The %RSD <2% for all analytes, confirming consistency (Tables 6.4.1–6.4.2).

Table 10; Interday precision data of pregabalin

Drug Name: pregabalin				
S. No.	Concentration (ppm)	Area	Std. Deviation	%RSD
DAY 1	500 PPM	1779584	17288.57766	0.98
	500 PPM	1746828		
	500 PPM	1772796		
DAY 2	500 PPM	1732821	21891.16398	1.27
	500 PPM	1700481		
	500 PPM	1742210		
DAY 3	500 PPM	1743002	18487.80164	1.07
	500 PPM	1711596		
	500 PPM	1744200		

	Range of % RSD	**0.95-1.20**

Table 11; Interday precision data of Methylcobalamin

Drug Name: methylcobalamin				
S. No.	Concentration (ppm)	Area	Std. Deviation	%RSD
DAY 1	10 ppm	7655260	87842.73368	1.15
	10 ppm	7551673		
	10 ppm	7726354		
DAY 2	10 ppm	7621058	68018.18277	0.90
	10 ppm	7593929		
	10 ppm	7492049		
DAY 3	10 ppm	7617772	7414.895167	0.10
	10 ppm	7603526		
	10 ppm	7614217		
	Range of % RSD			**0.10-1.15**

Table 12; Interday precision data of nortriptyline

Drug Name: nortriptyline				
S. No.	Concentration (ppm)	Area	Std. Deviation	%RSD
DAY 1	70 PPM	8474339	37729.82335	0.44
	70 PPM	8524260		
	70 PPM	8450294		
DAY 2	70 PPM	8524400	130883.1231	1.55
	70 PPM	8551881		
	70 PPM	8312697		
DAY 3	70 PPM	8599030	90934.32601	1.07
	70 PPM	8421055		
	70 PPM	8542459		
	Range of % RSD			**0.44-1.55**

2.6 Linearity studies of nortriptyline, pregabalin and methylcobalamin

The HPLC method demonstrated high linearity with $R^2 \approx 0.999$ for Nortriptyline, Pregabalin, and Methylcobalamin, confirming proportionality to concentration. LOD and LOQ, calculated from standard deviation and slope, were <5 µg/ml, ensuring high sensitivity for detecting low drug concentrations in pharmaceuticals and biological fluids.

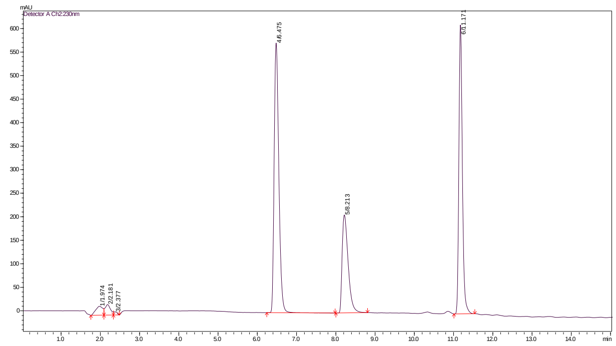

Figure 11; calibration studies; 1st calibration study of selected neuropathic pain inhibitors

Table 13; 1st calibration data of selected neuropathic pain inhibitors

Peak#	Ret. Time	Area	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
2	2.181	214389	1.9052	670.986	0.485	0.105	--	0
3	2.377	39051	0.347	41.798	0.216	0.204	--	1.943
Pregabalin	6.475	4244256	42.16	13194.37	4.834	2.281	1.389	11.176
Methyl-cobalamin	8.213	1813509	19.6704	13649.7	6.861	3.161	1.757	1.386
Nortriptyline	11.171	3800308	33.7716	66847.53	13.031	4.66	1.316	1.474

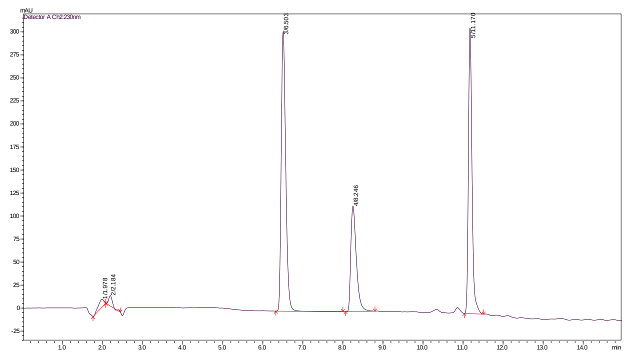

Figure 12; calibration studies; 2nd calibration study of selected neuropathic pain inhibitors

Table 14; 2nd calibration data of selected neuropathic pain inhibitors

Peak#	Ret. Time	Area	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
2	2.184	68731	1.2335	2398.867	0.9	0.104	1.125	0
Nortriptyline	6.503	2371789	42.5643	14698.56	21.986	2.288	1.366	21.963
Pregabalin	8.246	1103096	19.7963	16809.03	7.433	3.169	1.671	1.385

Methyl-cobalamin	11.17	1939644	34.809	67053.03	13.7	4.648	1.357	1.467

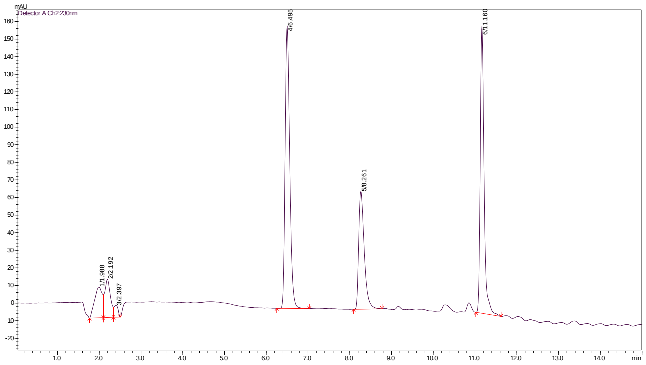

Figure 13; calibration studies; 3rd calibration study of selected neuropathic pain inhibitors

Table15; 3rd calibration data of selected neuropathic pain inhibitors

Peak#	Ret. Time	Area	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
2	2.192	207713	6.154	708.211	0.473	0.102	--	0
3	2.397	44359	1.3143	428.936	0.518	0.206	--	2.009
Nortriptyline	6.495	1235012	36.5905	15000.78	12.141	2.267	1.346	11.026
Pregabalin	8.261	620368	18.38	18108.53	7.717	3.155	1.648	1.392
Methyl-cobalamin	11.16	1042072	30.8741	67609.9	13.895	4.613	1.51	1.462

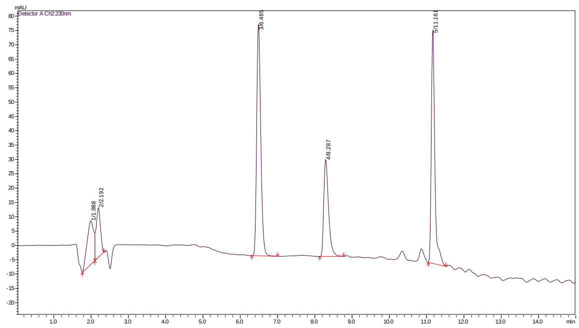

Figure 14; calibration studies; 4th calibration study of selected neuropathic pain inhibitors

Table 16; 4th calibration data of selected neuropathic pain inhibitors

Peak#	Ret. Time	Area	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.988	189750	10.6551	314.92	--	0	--	0
2	2.192	140958	7.9152	1026.387	0.567	0.103	--	0
Nortriptyline	6.485	614568	34.51	15415.99	17.79	2.263	1.326	21.995
Pregabalin	8.287	302011	16.9589	19673.68	8.096	3.17	1.582	1.401
Methyl-cobalamin	11.161	533553	29.9607	66415.75	14.034	4.616	1.762	1.456

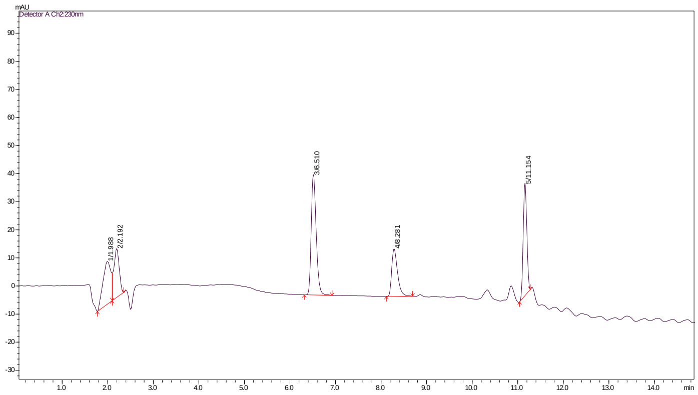

Figure 15; calibration studies; 5th calibration study of selected neuropathic pain inhibitors

Table 17; 5th calibration data of selected neuropathic pain inhibitors

Peak#	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.988	187312	15281	18.2289	323.661	--	0	--	0
2	2.192	142507	17407	13.8686	1001.023	0.568	0.103	--	0
Nortriptyline	6.51	324908	42810	31.6195	15555.81	17.771	2.275	1.327	22.15
Pregabalin	8.281	150585	16910	14.6547	19321.43	7.922	3.165	1.55	1.392
Methyl-cobalamin	11.154	222244	40093	21.6284	76004.19	14.36	4.611	1.123	1.457

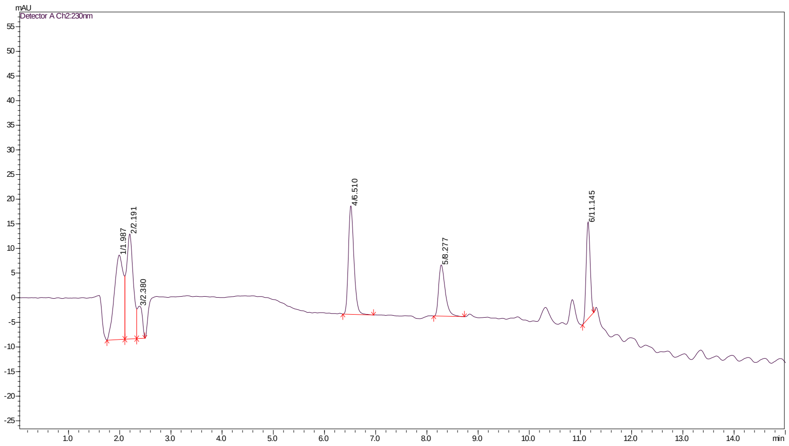

Figure 13; calibration studies; 6th calibration study of selected neuropathic pain inhibitors

Table 18; 6th calibration data of selected neuropathic pain inhibitors

Peak#	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation

1	1.987	213831	17163	25.9212	246.205	--	0	--	0
2	2.191	200290	21321	24.2797	726.781	0.492	0.103	--	0
3	2.38	44049	6559	5.3397	276.338	0.421	0.198	--	1.925
Pregabalin	6.51	166093	22036	20.1343	16037.66	10.611	2.277	1.319	11.492
Methyl-cobalamin	8.277	92946	10401	11.2672	19391.49	7.973	3.167	1.567	1.391
Nortriptyline	11.145	107718	19750	13.0579	77418.59	14.409	4.61	1.127	1.456

Table 19; Linearity data of pregabalin

Name of Drug: Pregabalin		
S. No.	Concentration ($\mu g.mL^{-1}$)	Area
1	250	4744256
2	125	2371789
3	62.5	1235012
4	31.25	614568
5	15.625	324908
6	7.8125	166093
Regression Equation		y=18854x + 29470
Correlation coefficient (R^2)		0.999
Std. error of intercept		9341.215421
Std. Dev. Of intercept		22881.21136
LOQ		12.14
LOD		3.64

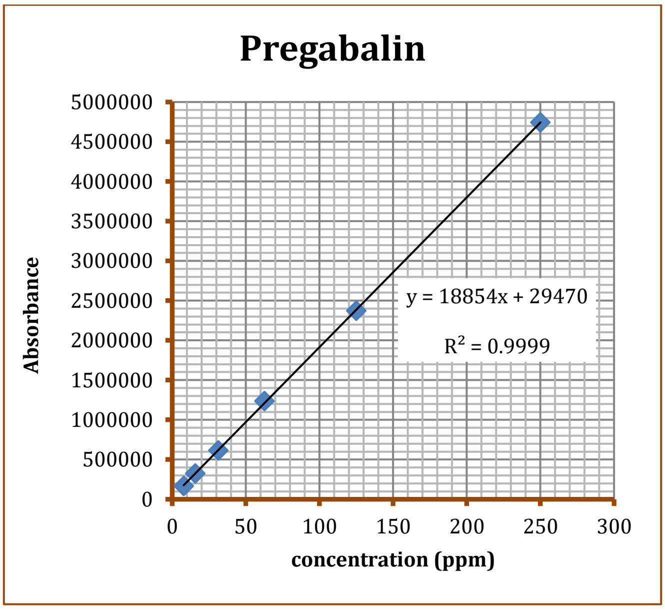

Fig. 17; Calibration curve of pregabalin

Table 20; Linearity data of methylcobalamin

Name of Drug; methylcobalamin		
S. No.	Concentration (µg.mL^{-1})	Area
1	10	2213509
2	5	1103096
3	2.5	620368
4	1.25	302011
5	0.625	150585
6	0.3125	74946
Regression Equation		y = 219026x + 25406
Correlation coefficient (R^2)		0.9991
Std. error of intercept		15723.04248
Std. Dev. Of intercept		38513.43128
LOQ		1.76
LOD		0.53

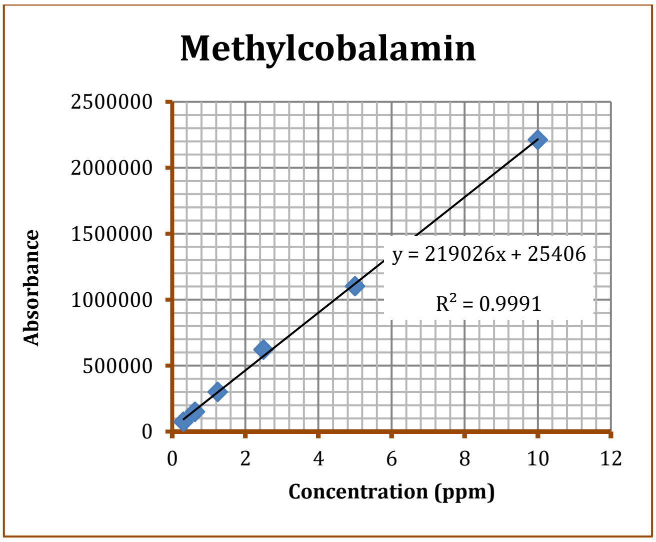

Fig. 18; Calibration curve of methylcobalamin

Table 21; Linearity data of Nortriptyline

Name of Drug; Nortriptyline

S. No.	Concentration ($\mu g.mL^{-1}$)	Area
1	35	3800308
2	17.5	1939644
3	8.75	1042072
4	4.375	533553
5	2.19	222244
6	1.09	107718
Regression Equation		y = 108374x + 29645
Correlation coefficient (R^2)		0.9991
Std. error of intercept		27421.25681
Std. Dev. Of intercept		67168.08729
LOQ		6.20
LOD		1.86

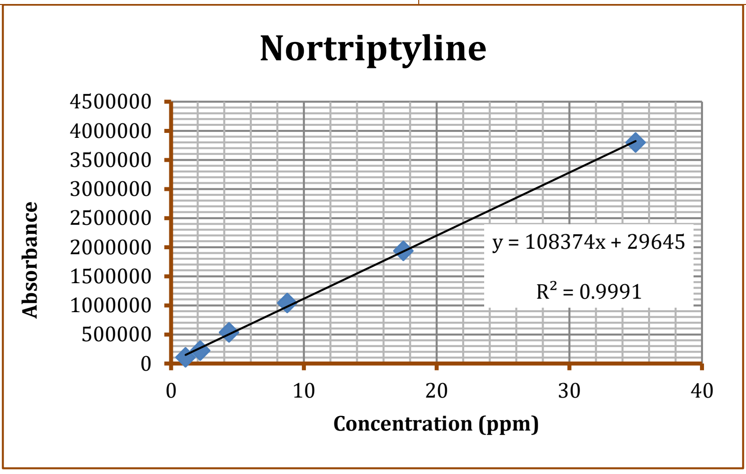

Fig. 19; Calibration curve of nortriptyline

2.7. Robustness for the chromatographic method

The HPLC method showed robustness. It stayed stable despite small changes in flow rate (1.0 ± 0.1 mL/min), organic modifier (70% ± 2%), and wavelength (230 ± 2 nm). Temperature, pH, eluent composition, and injection volume changes had little effect on capacity factor (k'), resolution (Rs), tailing factor (Tf), separation factor, theoretical plates (N), and peak area. This guarantees the method's reliability.

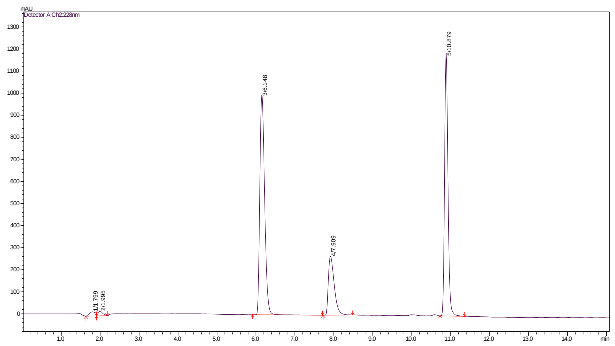

Figure 20; robustness study; effect of flow rate 1.1ml/min

Figure 22; robustness study; effect of flow rate 1.1ml/min

Peak#	Ret. Time	Area	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
2	1.995	188697	0.9964	629.707	0.524	0.109	--	0
Nortriptyline	6.148	8577255	45.2895	11081.89	15.055	2.417	1.375	22.165
Pregabalin	7.909	2779315	14.6753	12846.54	6.87	3.396	1.789	1.405
Methyl-cobalamin	10.879	7184350	37.9347	65803.49	13.236	5.047	1.266	1.486

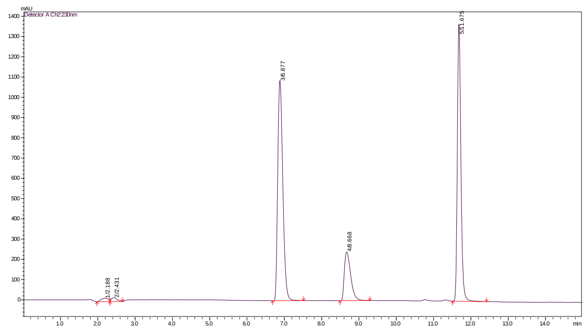

Figure 21; robustness study; effect of flow rate 0.9 ml/min

Figure 23; robustness study; effect of flow rate 0.9 ml/min

Peak#	Ret. Time	Area	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
2	2.431	215481	0.9475	771.121	0.533	0.111	--	0
Nortriptyline	6.877	10294635	45.2646	11541.11	14.668	2.143	1.378	19.286
Pregabalin	8.668	2854281	12.55	12187.7	6.282	2.962	1.691	1.382
Methyl-cobalamin	11.675	9128236	40.136	64892.6	12.091	4.336	1.312	1.464

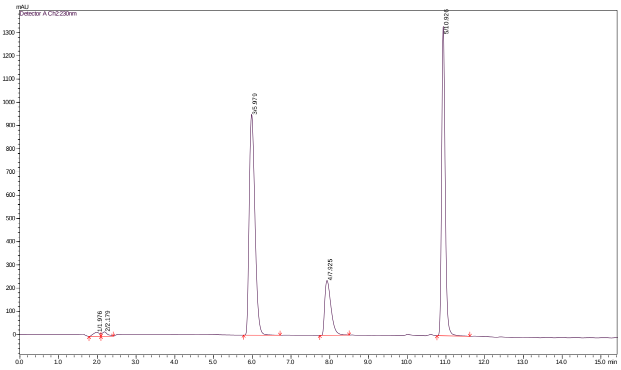

Figure 22; robustness study; effect of solvent B composition (32%)

Figure 24; robustness study; effect of solvent B composition (32%)

Peak#	Ret. Time	Area	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
2	2.179	185306	0.8712	753.953	0.519	0.103	--	0
Nortriptyline	5.979	9759039	45.8792	7435.225	12.776	2.025	1.389	19.732
Pregabalin	7.925	2643436	12.4273	11365.15	6.773	3.01	1.779	1.486
Methyl-cobalamin	10.926	8477878	39.8562	62174.45	12.698	4.528	1.295	1.504

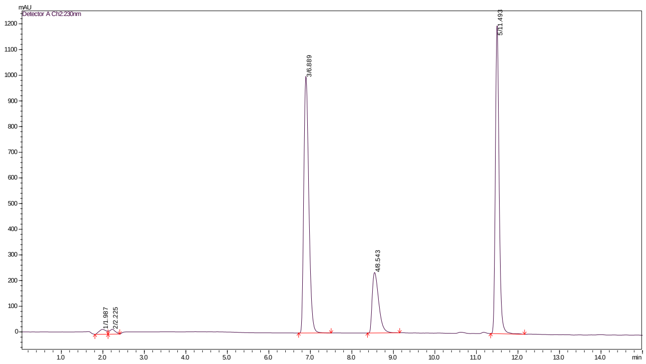

Figure 23; robustness study; effect of solvent B composition (28%)

Figure 25; robustness study; effect of solvent B composition (28%)

Peak#	Ret. Time	Area	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
2	2.225	184998	0.9763	723.342	0.623	0.12	--	0
Nortriptyline	6.889	8406639	44.365	14379.89	16.639	2.468	1.373	20.607
Pregabalin	8.543	2569392	13.5596	13991.48	6.376	3.3	1.747	1.337
Methyl-cobalamin	11.493	7557641	39.8845	69723.1	12.744	4.785	1.293	1.45

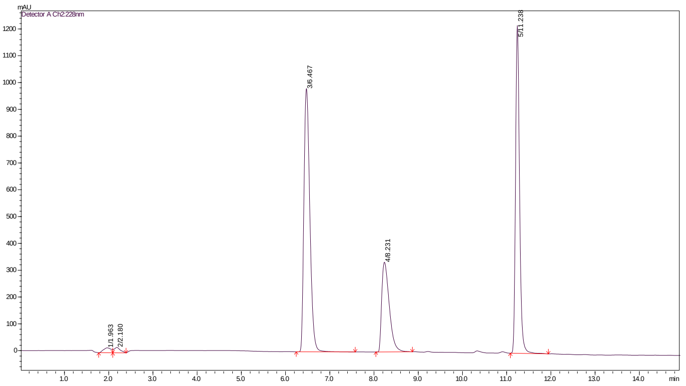

Figure 24 Robustness study; effect of wavelength 232 nm

Figure 26; robustness study; effect of wavelength 232 nm

Peak#	Ret. Time	Area	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.963	225604	1.0621	298.505	--	0	--	0
2	2.18	195236	0.9192	703.38	0.556	0.111	--	0
Nortriptyline	6.467	9130377	42.9855	10626.26	14.787	2.295	1.4	20.693
Pregabalin	8.231	3982768	18.7507	10852.93	6.223	3.194	1.801	1.392
Methyl-cobalamin	11.238	7706621	36.2825	65750.36	12.237	4.726	1.297	1.48

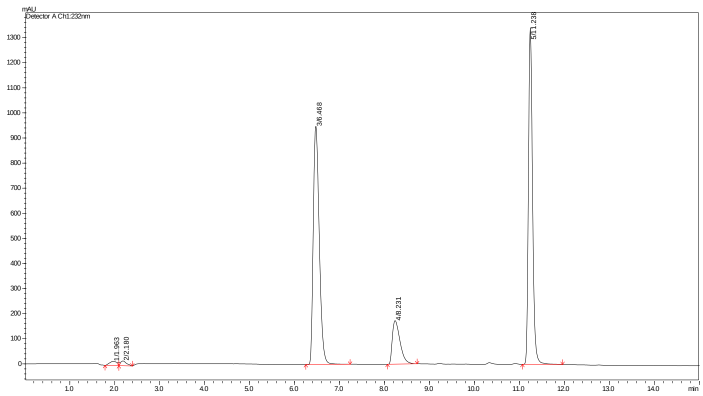

Figure 22; robustness study; effect of wavelength 228 nm

Figure 27; robustness study; effect of wavelength 228 nm

Peak#	Ret. Time	Area	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.963	225604	1.0621	298.505	--	0	--	0
2	2.18	195236	0.9192	703.38	0.556	0.111	--	0
Nortriptyline	6.467	9130377	42.9855	10626.26	14.787	2.295	1.4	20.693
Pregabalin	8.231	3982768	18.7507	10852.93	6.223	3.194	1.801	1.392
Methyl-cobalamin	11.238	7706621	36.2825	65750.36	12.237	4.726	1.297	1.48

Table No. 28; Robustness data of Nortriptyline

Variables of robustness	Nortriptyline			
	Retention time t_R (min)	Ret. factor (k')	Tailing factor (T_f)	Th. Plates (N)
Flow rate (+0.1 ml/min)	6.14	2.417	1.375	11081
Flow rate (- 0.1 ml/min)	6.87	2.143	1.378	11541
Solvent B composition (+2%)	5.97	2.02	1.38	7435
Solvent B composition (-2%)	7	3.16	1.35	16705
Wavelength (+2 nm)	6.6	2.97	1.35	14597
Wavelength (-2 nm)	6.6	2.97	1.35	14597
Average	6.63	2.78	1.36	
Standard deviation	±0.33	±0.49	±0.01	

Table No. 29; Robustness data of Pregabalin

Variables of robustness	Pregabalin				
	t_R (min)	k'	Tf	R_S	N
Flow rate (+0.1 ml/min)	7.909	3.396	1.489	6.87	12846
Flow rate (- 0.1 ml/min)	8.668	2.962	1.691	6.282	12187
Solvent B composition (+2%)	7.925	3.01	1.77	6.77	11365
Solvent B composition (-2%)	8.54	3.33	1.74	6.37	13991
Wavelength (+2 nm)	8.23	3.19	1.80	6.23	10852
Wavelength (-2 nm)	8.23	3.19	1.80	6.22	10852
Average	8.25	3.18	1.72	6.46	
Standard deviation	± 0.31	± 0.17	± 0.11	± 0.29	

Table No. 30; Robustness data of Methyl-cobalamin

Variables of robustness	Methyl-cobalamin				
	tR (min)	k'	Tf	R_S	N
Flow rate (+0.1 ml/min)	10.879	5.047	1.266	13.236	65803.

Flow rate (- 0.1 ml/min)	11.675	4.336	1.312	12.091	64892
Solvent B composition (+2%)	10.926	4.528	1.295	12.698	62174
Solvent B composition (-2%)	11.493	4.785	1.293	12.744	69723
Wavelength (+2 nm)	11.238	5.95	1.3	12.237	65750
Wavelength (-2 nm)	11.57	4.72	1.297	12.237	65750
Average	11.30	4.89	1.29	12.54	
Standard deviation	± 0.34	± 0.57	± 0.01	± 0.43	

Increasing the flow rate (+0.1 mL/min) slightly reduced tR values, while decreasing it (-0.1 mL/min) slightly extended them. Small changes in organic solvent concentration (30% ± 2%) and wavelength (230 ± 2 nm) did not affect the retention order or peak sensitivity. Robustness studies confirmed minimal variation, meeting ICH guidelines (Table 6.9).

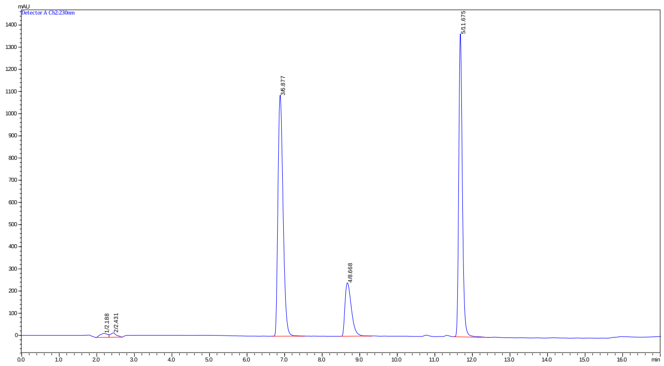

Figure 26; Drug accuracy study; simultaneous estimation of selected standards

Figure 31; Drug accuracy study; simultaneous estimation of selected standards

Peak#	Ret. Time	Area	Area%	T.Plate#	Tailing F.	Resolution	k'	Separation
1	1.786	102248	0.4349	345.587	0.812	--	0	0
2	2.188	267988	1.1398	279.933	--	0.886	0.225	0
3	2.431	225420	0.9587	747.865	--	0.553	0.361	1.604
pregabalin	6.877	10418256	44.31	11534.16	1.378	14.538	2.851	7.893
methylcobalamin	8.668	3798750	16.1565	12296.86	1.693	6.296	3.854	1.352
nortriptyline	11.675	8699529	37.0001	65630.41	1.315	12.151	5.537	1.437

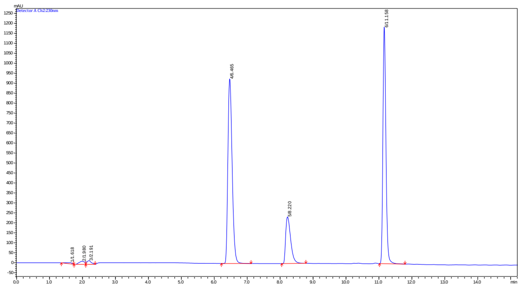

Figure 27; Drug accuracy study; simultaneous estimation of marketed formulation

Figure 32; Drug accuracy study; simultaneous estimation of marketed formulation

Peak#	Ret. Time	Area	Area%	T.Plate#	Tailing F.	Resolution	k'	Separation
1	1.618	67213	0.3593	450.321	0.697	--	0	0
2	1.98	185515	0.9918	275.256	--	0.925	0.224	0
3	2.191	171431	0.9165	803.476	--	0.536	0.354	1.582
pregabalin	6.465	9726197	44.5141	11193.77	1.358	15.442	2.996	8.462
methylcobalamin	8.22	2462220	13.1637	13803.12	1.743	6.694	4.08	1.362
nortriptyline	11.158	7992049	40.0545	64918.39	1.286	12.911	5.896	1.445

2.9; Accuracy studies of marketed formulation

We conducted accuracy studies at 80%, 100%, and 120% concentrations. Each concentration was injected three times. We estimated Nortriptyline, Methylcobalamin, and Pregabalin from market products (see Table 7.34). We determined drug recovery using the calibration curve, Y-intercept, and slope. We also compared it with reference standards. Recovery rates were 100.4–100.7% for Nortriptyline. For Methylcobalamin and Pregabalin, they ranged from 100–105%. All these rates fall within the ICH-recommended range of 90–110%. The %RSD remained <2%, confirming method accuracy for simultaneous quantification. (Table 33: Accuracy data of Pregabalin).

Drug Name: pregabalin			Drug content: 75 mg		Marketed formulation: Cobivital NTM Tablet		
Std. conc. (%)	Std. (ppm)	Peak area	Drug (%)	Drug (ppm)	Peak area	Avg. peak area	Drug Rec. (%)
100%	500 ppm	10418256	80	400	7701159	7705911	92.46
				400	7710663		
			100	500	9726197	9750663	93.59

				500	9775129		
			120	600	12093061	12275686	98.19
				600	12458311		
Drug recovery Range (%) as per ICH = 100±10%				92.46 % - 98.19%			

Table 34; Accuracy data of methylcobalamin

Drug Name: methylcobalamin			Drug content: 1.5 mg		Marketed formulation; Cobivital NTM Tablet		
Std. conc. (%)	Std. (ppm)	Peak area	Drug (%)	Drug (ppm)	Peak area	Avg. peak area	Drug Rec. (%)
100%	10 ppm	3798750	80	8	2079419	2088879.5	68.74
				8	2098340		
			100	10	2462220	2498493	65.77
				10	2534766		
			120	12	2840759	2820872.5	61.88
				12	2800986		
Drug recovery Range (%) as per ICH = 100±10%				61.88% - 68.74%			

Table 35; Accuracy data of nortriptyline

Drug Name: nortriptyline			Drug content: 10 mg		Marketed formulation; Cobivital NTM Tablet		
Std. conc. (%)	Std. (ppm)	Peak area	Drug (%)	Drug (ppm)	Peak area	Avg. peak area	Drug Rec. (%)
100%	70	8699529	80	56	6378862	6348042.5	91.21
				56	6317223		
			100	70	7992049	8004105.5	92.01
				70	8016162		
			120	84	10242973	10248191	98.17
				84	10253409		
Drug recovery Range (%) as per ICH = 100±10%				91.21% - 98.17%			

Conclusion:

This study created and tested a way to measure Nortriptyline, Pregabalin, and Methylcobalamin together. We used RP-HPLC and followed ICH guidelines. The C8 column worked better than regular C18 columns. It had improved separation and retention, which helped in accurately distinguishing analytes. The method showed great linearity ($R^2 \approx 0.999$), precision (RSD < 2%), and accuracy (recovery 100–105%). It also showed strong reliability. This makes it suitable for pharmaceutical and bioanalytical use. The system suitability parameters were also acceptable. The capacity factor (k'), resolution (Rs), and tailing factor (Tf) all met the ICH standards. These findings show that the RP-HPLC method effectively estimates neuropathic pain inhibitors in drugs. It is also reproducible.

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