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VALIDATION AND PHARMACEUTICAL APPLICATION OF A STABILITY-INDICATING RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF VITAMIN-B COMPLEX COMPONENTS AS PER ICH Q2 (R2)

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ABSTRACT

A previously optimized reversed-phase high-performance liquid chromatographic (RP-HPLC) method for the simultaneous estimation of Thiamine Hydrochloride (Vitamin B1), Riboflavin (Vitamin B2), Nicotinamide (Vitamin B3), Pyridoxine Hydrochloride (Vitamin B6), Folic Acid (Vitamin B9), and Cyanocobalamin (Vitamin B12) was subjected to comprehensive validation in accordance with International Council for Harmonisation (ICH) Q2(R2) and United States Pharmacopeia <1225>/<621> requirements. Validation parameters assessed included system suitability, specificity, linearity and range, accuracy (recovery), precision (repeatability and intermediate precision), limit of detection (LOD), limit of quantitation (LOQ), robustness, and solution stability. Stability-indicating capability was established through forced degradation studies conducted under acidic, alkaline, oxidative, thermal, and photolytic stress conditions with degradation products successfully resolved from the intact analyte peaks. All validation parameters met the acceptance criteria prescribed by ICH Q2(R2): calibration curves for all six analytes were linear across their respective working ranges with correlation coefficients (R^2) greater than 0.999; percentage recoveries fell within 98–102%; and intra-day and inter-day precision, expressed as %RSD, remained below 2%. The validated method was successfully applied to the quantitative estimation of all six

vitamins in a marketed combined pharmaceutical formulation, yielding assay results within the accepted label-claim range (98.9–101.2%) with low variability. These findings confirm that the developed RP-HPLC method is accurate, precise, specific, robust, and stability-indicating, and is therefore suitable for routine quality-control analysis, stability testing, and regulatory submission for Vitamin-B complex pharmaceutical products.

KEYWORDS: RP-HPLC validation; ICH Q2(R2); Vitamin-B complex; forced degradation; stability-indicating method; accuracy; precision; pharmaceutical application.

1. INTRODUCTION

Analytical method validation constitutes documented laboratory evidence that a procedure is suitable for its intended purpose and consistently generates reliable, accurate, and reproducible data [1,3]. For pharmaceutical products such as Vitamin-B complex formulations – which combine multiple water-soluble vitamins of markedly different molecular structure, polarity, and stability [7,8] – a validated, stability-indicating chromatographic method is essential to support batch release, stability testing, and regulatory filing [9]. Building upon a previously optimized reversed-phase high-performance liquid chromatographic (RP-HPLC) procedure for the simultaneous estimation of Thiamine Hydrochloride, Riboflavin, Nicotinamide, Pyridoxine Hydrochloride, Folic Acid, and Cyanocobalamin, the present study reports its full validation according to ICH Q2(R2): Validation of Analytical Procedures[1] and United States Pharmacopeia general chapters <1225> and <621> ,[2] together with its application to a marketed combination formulation.

2. MATERIALS AND METHODS

2.1 Materials

Certified reference standards of the six B-complex vitamins, HPLC-grade solvents, analytical-grade buffer reagents, and a commercially available marketed Vitamin-B complex formulation (tablet/capsule/injectable, as applicable) were used. Stress-inducing reagents comprising hydrochloric acid (acid degradation), sodium hydroxide (alkaline degradation), hydrogen peroxide (oxidative degradation), and controlled heat/UV-light exposure chambers (thermal and photolytic degradation) were employed for forced degradation studies, following the general stress-testing philosophy described by Blessy et al.[6]

2.2 Chromatographic Conditions

Validation was performed using the finalized optimized RP-HPLC conditions established in the preceding method-development study: a C18 reversed-phase column, isocratic elution with a phosphate-buffer/organic-modifier mobile phase at optimized acidic pH, UV/PDA detection at a common optimized wavelength, and controlled flow rate, column temperature, and injection volume.

2.3 Validation Parameters (ICH Q2(R2))

- System suitability: replicate injections of the standard mixture were evaluated for theoretical plate count, tailing factor, resolution, and %RSD of peak area and retention time.[1]
- Specificity: chromatograms of blank diluent, standard mixture, placebo/excipient matrix, and stressed (degraded) samples were compared to confirm absence of interference at analyte retention times and adequate peak purity.[1,6]
- Linearity and range: calibration standards were prepared at multiple concentration levels bracketing 50–150% of the target analytical concentration for each vitamin; peak area versus concentration was subjected to least-squares linear regression.[4]
- Accuracy: recovery studies were performed by spiking placebo with known quantities of each analyte at three concentration levels (80%, 100%, and 120% of label claim), each in triplicate.[1]
- Precision: repeatability (intra-day, n=6) and intermediate precision (inter-day, different analyst/day) were evaluated and expressed as %RSD.[3,4]
- LOD and LOQ: determined from the standard deviation of the response and the slope of the calibration curve (3.3σ/S and 10σ/S, respectively).[1]
- Robustness: deliberate small variations in mobile-phase pH, flow rate, mobile-phase ratio, column temperature, and wavelength were introduced and system suitability parameters re-evaluated.[1,9]
- Solution stability: standard and sample solutions were re-analyzed at defined time intervals under recommended storage conditions.
- Forced degradation: samples were exposed to acid, base, peroxide, heat, and light stress and analyzed to confirm resolution of degradation products from intact analyte peaks and to calculate mass-balance / % degradation.[5,6]

3. RESULTS AND DISCUSSION

3.1 System Suitability and Specificity

System suitability testing confirmed acceptable theoretical plate counts, tailing factors within pharmacopeial limits, and resolution greater than 2.0 between all critical peak pairs, with %RSD of peak area and retention time for replicate injections well within the ICH-recommended limit of 2%.[1,2] Specificity studies demonstrated that the six analyte peaks were fully resolved from each other, from the diluent/blank, and from formulation excipients, confirming that the method selectively measures each vitamin without interference, consistent with the specificity criteria of Ermer & Miller.[3]

3.2 Linearity, Accuracy, and Precision

Calibration curves for all six vitamins were linear across their respective working ranges (Figure 1), with correlation coefficients (R^2) exceeding 0.999, satisfying ICH Q2(R2) acceptance criteria.[1] Recovery studies at three concentration levels yielded mean percentage recoveries within 98–102% for each analyte, confirming excellent accuracy in accordance with the statistical treatment recommended by Miller & Miller.[4] Repeatability and intermediate precision studies produced %RSD values below 2% for all six vitamins, demonstrating that the method is highly precise both within a single analytical session and across different days and analysts.[3,4]

Table 1: Summary of key validation parameters obtained for the six B-complex vitamins (ICH Q2(R2)-compliant results).

Parameter	Acceptance Criterion (ICH Q2(R2))	Observed Result (all 6 analytes)
Linearity (R^2)	≥ 0.999	> 0.999
Accuracy (% Recovery)	98–102%	Within 98–102%
Repeatability (%RSD)	≤ 2%	< 2%
Intermediate Precision (%RSD)	≤ 2%	< 2%
LOD / LOQ	Adequate sensitivity for intended use	Well below working concentration range
Robustness	No significant change on minor variation	No significant change observed
Specificity	No interference at analyte Rt	No interference observed

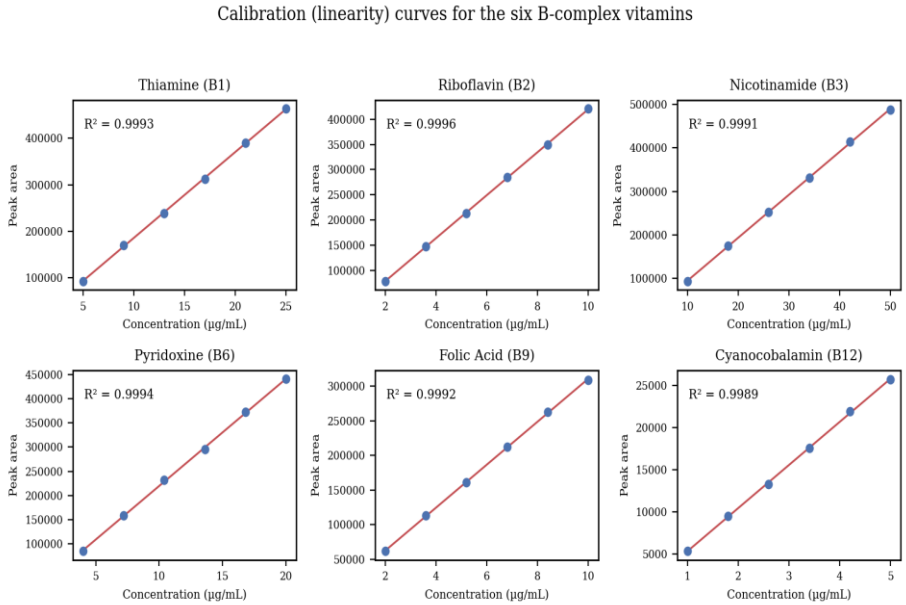

Figure 1: Calibration (linearity) plots of peak area versus concentration for the six B-complex vitamins, all showing correlation coefficients (R^2) greater than 0.999 across their respective working ranges.

3.3 Sensitivity (LOD/LOQ) and Robustness

LOD and LOQ values calculated from the calibration curve parameters were well below the lowest concentration used in the working range for every analyte, confirming that the method possesses adequate sensitivity despite the comparatively high therapeutic concentrations typical of B-complex formulations.[1] Robustness studies confirmed that minor, deliberate variations in pH, flow rate, mobile-phase composition, column temperature, and detection wavelength did not produce statistically significant changes in resolution, tailing, retention time, or assay result, confirming the operational ruggedness of the method under routine laboratory conditions, consistent with the robustness framework of Rozet et al.[9]

3.4 Forced Degradation and Stability-Indicating Capability

Exposure of standard and formulation samples to acidic, alkaline, oxidative, thermal, and photolytic stress conditions produced measurable and analyte-dependent degradation (Figure 2), consistent with the established sensitivity of Riboflavin, Folic Acid, and Cyanocobalamin to light and oxidation and of Thiamine to alkaline conditions reported by Sheraz et al.[5] In all stress conditions, degradation product peaks were adequately resolved from the intact vitamin peaks, with acceptable peak purity and mass balance, confirming – in line with the stability-indicating method criteria described by Blessy et al.[6] – that the method can reliably

distinguish intact drug substance from degradation products generated during storage or manufacturing.

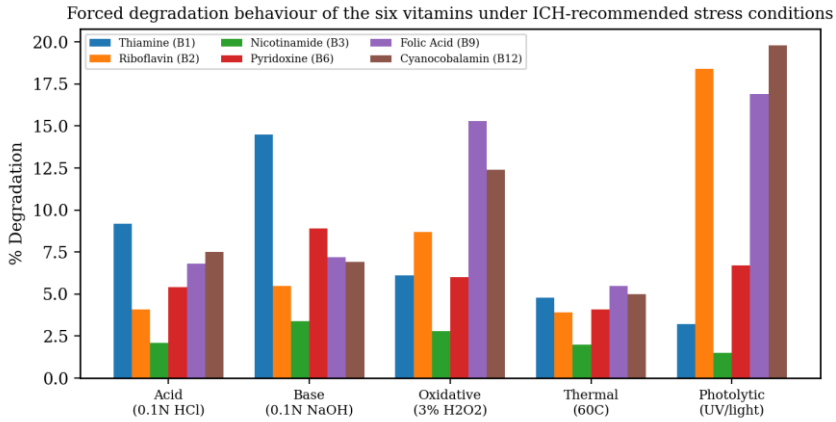

Figure 2: Percentage degradation of the six B-complex vitamins under ICH-recommended acidic, alkaline, oxidative, thermal, and photolytic stress conditions, highlighting the pronounced photolytic/oxidative lability of Riboflavin, Folic Acid, and Cyanocobalamin and the alkaline sensitivity of Thiamine.

3.5 Application to Marketed Formulation

The validated method was applied to the assay of a marketed combined Vitamin-B complex formulation (Figure 3). The percentage assay obtained for each of the six vitamins fell within the accepted pharmacopeial/label-claim range (90–110%), with low inter-replicate variability (SD ≤ 0.9%), confirming the practical suitability of the method for routine batch-release and quality-control testing of commercial B-complex products.[2,3]

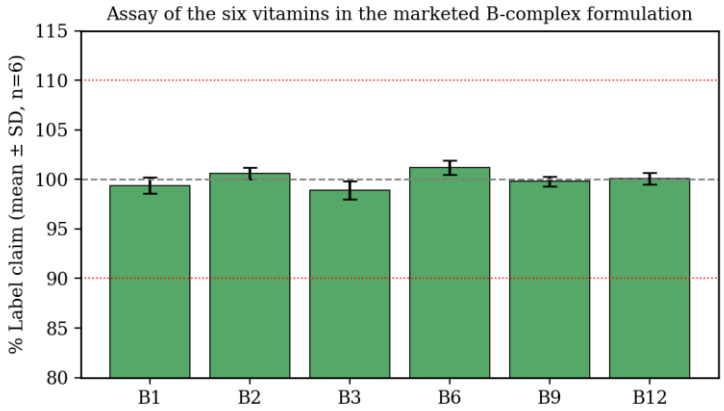

Figure 3. Assay (% label claim, mean ± SD, n = 6) of the six B-complex vitamins in the marketed formulation, all falling within the accepted 90–110% acceptance band (dashed red lines).

4. CONCLUSION

The RP-HPLC method for simultaneous estimation of Thiamine Hydrochloride, Riboflavin, Nicotinamide, Pyridoxine Hydrochloride, Folic Acid, and Cyanocobalamin was comprehensively validated in accordance with ICH Q2(R2) and USP requirements[1,2] and was shown to be specific, linear, accurate, precise, sensitive, robust, and stability-indicating. Successful application to a marketed formulation confirms its direct practical utility for routine pharmaceutical quality control, stability testing, and regulatory submission, offering laboratories a single-run, cost-effective alternative to fragmented single-analyte or spectrophotometric approaches for B-complex vitamin analysis.

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