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Analytical Methods for the Quantification of Pregabalin, Nortriptyline, and Vitamin B12 (Methylcobalamin) in Combined Dosage Forms: A Comprehensive Literature Review

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ABSTRACT

Neuropathic pain is increasingly managed with fixed-dose combinations that pair a gabapentinoid, a tricyclic antidepressant, and a neurotropic vitamin. Pregabalin, nortriptyline, and vitamin B12 (as methylcobalamin) represent one such rational triad, marketed together for diabetic peripheral neuropathy, post-herpetic neuralgia, and related conditions. Reliable quality control of this combination is complicated by a fundamental analytical obstacle: pregabalin possesses no useful UV chromophore and negligible native fluorescence, so it escapes direct spectrophotometric and many chromatographic detection schemes unless it is chemically derivatised. This review consolidates the published analytical literature on pregabalin, nortriptyline, and vitamin B12, individually and in combination with each other and with third-party drugs, with particular emphasis on the derivatisation strategies (ninhydrin, 1-fluoro-2,4-dinitrobenzene, p-dimethylaminobenzaldehyde, benzoyl chloride, diazotisation-coupling, and chloroformate reagents) that have been used to render pregabalin detectable. Spectrophotometric, HPLC, HPTLC, LC-MS/MS, GC-MS, titrimetric, and electrochemical approaches are surveyed for each drug and for the reported binary and ternary combinations. Only a small number of methods address the complete pregabalin-nortriptyline-methylcobalamin triad, and none of the identified ternary methods report a validated, stability-indicating, chromatographic assay published in a mainstream indexed pharmaceutical analytical journal that also incorporates a rigorously validated derivatisation step for pregabalin. This gap, together with the near-total absence of green-chemistry-assessed or Analytical Quality-by-Design (AQbD) approaches for this triad, defines a clear scope for future method development. The review concludes by outlining priority directions for a stability-indicating, derivatisation-based RP-HPLC or HPTLC method for the simultaneous estimation of pregabalin, nortriptyline, and methylcobalamin in combined tablet dosage forms.

Keywords: Pregabalin; Nortriptyline; Vitamin B12; Methylcobalamin; derivatisation; ninhydrin; chromophore; RP-HPLC; HPTLC; UV-spectrophotometry; fixed-dose combination; neuropathic pain; analytical method validation

1. INTRODUCTION

Neuropathic pain arising from diabetic peripheral neuropathy, post-herpetic neuralgia, spinal cord injury, and related nerve-damage conditions is notoriously difficult to control with a single pharmacological class. Contemporary treatment algorithms therefore favour rational polypharmacy: an anticonvulsant/gabapentinoid to dampen aberrant neuronal excitability, a tricyclic antidepressant to potentiate descending inhibitory pain pathways, and a neurotropic B-vitamin to support nerve repair and myelin regeneration (Apollo Pharmacy, n.d.; PlatinumRx, n.d.). Pregabalin, nortriptyline, and methylcobalamin (the biologically active coenzyme form of vitamin B12) are combined on precisely this logic, and fixed-dose combination (FDC) tablets containing all three actives are now widely marketed, particularly across the Indian pharmaceutical market, for diabetic neuropathy, post-herpetic neuralgia, fibromyalgia, and chronic lower-back or sciatic pain (Apollo Pharmacy, n.d.; Medicoverhospitals, n.d.; Truemeds, n.d.).

Clinical evidence supports the individual and combined use of these agents. Pregabalin, a structural analogue of gamma-aminobutyric acid (GABA), binds the alpha-2-delta subunit of voltage-gated calcium channels to reduce excitatory neurotransmitter release, and sustained-release pregabalin combined with methylcobalamin has demonstrated significant, rapid, and well-tolerated pain reduction in Indian real-world cohorts. Nortriptyline, a second-generation tricyclic antidepressant and the principal active metabolite of amitriptyline, augments serotonergic and noradrenergic descending inhibition and has separately been compared with vitamin B12 for painful diabetic neuropathy. Recognising the complementary mechanisms of the three agents, several manufacturers now co-formulate pregabalin, nortriptyline, and methylcobalamin as a single tablet to improve adherence and simplify dosing regimens (Medicoverhospitals, n.d.).

Robust quality control of any fixed-dose combination requires a validated analytical method capable of resolving and quantifying every active in the presence of the others and of the tablet excipients. For the pregabalin-nortriptyline-methylcobalamin triad this is analytically non-trivial for a reason that is specific to pregabalin: unlike nortriptyline (which carries a dibenzocycloheptene chromophore) and methylcobalamin (which carries an extensively conjugated corrin ring absorbing strongly through the visible region), pregabalin is an aliphatic gamma-amino acid analogue with no aromatic ring, no extended conjugation, and therefore negligible ultraviolet absorbance above 200 nm and negligible native fluorescence (Bali & Gaur, 2011; Gujral et al., 2009). A recent comprehensive review characterises this succinctly: pregabalin's 'aliphatic structure and absence of chromophoric groups' represents the central and recurring obstacle across two decades of method-development literature, despite the drug's otherwise favourable BCS Class I solubility and permeability profile (Varu, Malviya, et al., 2025). Consequently, any spectrophotometric or many chromatographic UV-based methods for pregabalin-containing formulations must either work around the problem (e.g., using very short wavelengths close to the solvent cut-off, which sacrifices selectivity) or convert pregabalin into a UV/visible-active derivative before measurement.

This review has three purposes. First, it consolidates the individual analytical literature for pregabalin, nortriptyline, and vitamin B12/methylcobalamin, with particular attention to the derivatisation chemistries developed to overcome pregabalin's lack of chromophore. Second, it surveys methods reported for each drug in combination with drugs other than the two partners considered here, to establish the breadth of validated chromatographic and spectroscopic platforms already available for adaptation. Third, and centrally, it reviews the limited body of work addressing the pregabalin-nortriptyline binary combination and the pregabalin-nortriptyline-methylcobalamin ternary combination, in order to identify concrete gaps and to propose a scope for future method development.

2. Overview of the Three Actives

2.1 Pregabalin

Pregabalin, (S)-3-(aminomethyl)-5-methylhexanoic acid, is a structural analogue of GABA and of the endogenous amino acid L-leucine. It is official in several pharmacopoeias and is used as an anticonvulsant, anxiolytic, and first-line agent for neuropathic pain by virtue of its high affinity for the alpha-2-delta auxiliary subunit of voltage-gated calcium channels (Gujral et al., 2009; Kasawar & Farooqui, 2010). Pharmaceutically it is a small, highly water-soluble, non-hygroscopic, single-enantiomer molecule that undergoes minimal hepatic metabolism, with more than 90% excreted unchanged in urine. Its principal analytical liability, discussed throughout this review, is the complete absence of a UV chromophore or fluorophore in its structure (Varu, Malviya, et al., 2025).

2.2 Nortriptyline Hydrochloride

Nortriptyline hydrochloride, chemically 3-(10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5-ylidene)-N-methyl-1-propanamine hydrochloride, is a second-generation tricyclic antidepressant and the major active metabolite of amitriptyline. Beyond depression it is used for neuropathic pain, migraine prophylaxis, and smoking cessation (Bagul et al., 2024). Structurally, nortriptyline carries a dibenzocycloheptene (tricyclic) chromophore that gives it strong, well-defined UV absorbance (lambda-max close to 239 nm in methanol), making it, in marked contrast to pregabalin, straightforward to detect directly by UV-spectrophotometry and conventional HPLC-UV (Patel, 2020).

2.3 Vitamin B12 (Methylcobalamin/Cyanocobalamin)

Vitamin B12 is a cobalt-centred corrinoid essential for myelin synthesis, DNA methylation, and red-blood-cell maturation. Methylcobalamin, the coenzyme form most often used in neuropathy formulations, and cyanocobalamin, the more chemically stable form used in most multivitamin products, both possess an extensively conjugated corrin-ring chromophore that absorbs strongly in the UV-visible region (around 265, 361, and 550 nm), which makes vitamin B12 congeners readily amenable to direct UV-visible spectrophotometry and to HPLC with UV/DAD detection despite the very low doses (micrograms) typically present in tablets (Galande et al., 2010).

3. Analytical Methods Reported for Pregabalin

3.1 Methods Not Requiring Derivatisation

Because no pharmacopoeial monograph existed for pregabalin for many years, early workers were forced to develop original methods from first principles (Gujral et al., 2009). A number of RP-HPLC methods exploit pregabalin's very weak absorbance close to the ultraviolet cut-off of common mobile-phase components, typically reading at 200-210 nm. Kasawar and Farooqui (2010) reported an RP-HPLC method on a Hypersil BDS C8 column with phosphate buffer (pH 6.9)-acetonitrile (95:5) and photodiode-

array detection, explicitly noting that pregabalin quantitation without pre-column derivatisation had not previously been achieved. Gujral et al. (2009) validated a direct UV-spectrophotometric method at 210 nm, linear over 0.5-5.0 micrograms/mL, and applied it to bulk drug, formulations, and human urine. Related RP-HPLC methods have used Inertsil ODS-3V or ODS Hypersil C18 columns with disodium hydrogen phosphate/acetonitrile/methanol or methanol-acetonitrile-dipotassium hydrogen orthophosphate mobile phases, again detecting at 210 nm, and have been extended to related-substance and stability-indicating assays. A non-aqueous potentiometric titrimetric method using perchloric acid and crystal violet indicator has also been validated for pregabalin bulk-drug assay, circumventing the chromophore problem entirely by avoiding optical detection. At the bioanalytical end of the spectrum, LC-MS/MS methods (monitoring, for example, the 160.2 to 55.1 m/z transition) allow pregabalin to be quantified in plasma without any derivatisation because mass-selective detection does not require a chromophore, and such methods are now the preferred approach for pharmacokinetic and clinical-toxicology work, albeit at instrumentation cost and complexity that make them unsuitable for routine tablet-QC laboratories.

3.2 Derivatisation-Based Methods for Pregabalin

The larger and analytically more interesting body of pregabalin literature involves deliberate chemical derivatisation of the drug's primary amine group to install a chromophore, fluorophore, or mass-spectrometrically favourable moiety. A recent critical review classifies these into titrimetric, spectroscopic (UV-Vis, fluorescence, infrared), electrochemical, chromatographic (HPTLC, HPLC, GC), and mass-spectrometric derivatisation strategies, reporting detection limits spanning from about 19 micrograms/mL down to 4.9 nanograms/mL depending on the reagent and technique used (Varu, Malviya, et al., 2025).

- Ninhydrin derivatisation. Pregabalin's primary amine reacts with ninhydrin (triketohydrindene hydrate) to generate Ruhemann's Purple, a deep violet chromogen with absorbance maxima variously reported between 402 and 580 nm depending on reaction conditions and solvent. Bali and Gaur (2011) first reported this reaction for pregabalin, heating the reagents at 70-75 degrees C for 20 minutes to develop the colour reproducibly, and validated the method for pure drug and capsules. Mutalabisin et al. (2021) subsequently coupled ninhydrin pre-column derivatisation to HPLC-UV detection at 569 nm, reporting linearity from 50 to 600 micrograms/mL and mean recovery within 100 +/- 2%, describing it as the first HPLC application of ninhydrin derivatisation for pregabalin. The same chemistry has since been extended to combination products: a green HPLC-DAD/HPTLC method for pregabalin-amitriptyline mixtures scanned the ninhydrin-pregabalin adduct at 580 nm while amitriptyline was read directly at 215 nm, and an HPTLC method for nortriptyline-pregabalin combined tablets used post-chromatographic ninhydrin derivatisation to visualise and quantify the pregabalin band at Rf 0.279, while nortriptyline (Rf 0.523) required no derivatisation because it does not react with ninhydrin, a fact exploited for selectivity (Harkhani et al., 2023). Most recently, a ninhydrin-mediated spectrophotometric assay for pregabalin-duloxetine mixtures again used the Ruhemann's Purple reaction (570-580 nm) for pregabalin while duloxetine was read directly at 230 nm.

- 1-Fluoro-2,4-dinitrobenzene (Sanger's reagent). Pregabalin's amine undergoes nucleophilic aromatic substitution with 1-fluoro-2,4-dinitrobenzene (FDNB) to form a UV-active dinitrophenyl derivative measurable near 360 nm; this chemistry has been used both for HPLC-UV quantitation in bulk drug and dosage forms and for bioanalytical plasma assays (Walash et al., 2011).

- p-Dimethylaminobenzaldehyde (PDAB) condensation. Pregabalin condenses with PDAB under acidic conditions to give a coloured Schiff-base-type complex with maximum absorption at 395.8 nm; this reagent has been applied in spectrophotometric assays validated against Indian Pharmacopoeia reference methods.

- Benzoylation. Reaction of the primary amine with benzoyl chloride introduces an aromatic benzoyl chromophore, allowing benzoylated pregabalin to be read directly at 225 nm; this approach was used specifically to enable simultaneous Q-absorbance-ratio UV analysis of pregabalin alongside nortriptyline, which is itself read at 239 nm (its native lambda-max) and at 235 nm (the iso-absorptive point) (Patel, 2020).

- Diazotisation and azo-coupling. A recently reported method diazotised pregabalin's amine and coupled the product with beta-naphthol at 2-5 degrees C to generate an azo dye with lambda-max at 355 nm, achieving very low limits of detection (0.001 micrograms/mL) and quantitation (0.003 micrograms/mL), reported as superior to previously published derivatisation approaches for sensitivity (Malviya et al., 2026).

- Chloroformate derivatisation for GC-MS and LC-MS/MS. For bioanalytical and forensic-toxicology applications, pregabalin has been derivatised with ethyl chloroformate or n-propyl chloroformate/n-propanol prior to GC-MS or LC-MS/MS analysis, improving chromatographic behaviour and ionisation efficiency for a molecule that is otherwise poorly retained and poorly ionised; such methods have been applied to dried blood spots and dried plasma spots as well as conventional plasma.

Taken together, this body of work confirms that a working analyst faced with pregabalin has, in effect, two families of options: avoid optical detection altogether (titrimetry, mass spectrometry, or very-low-wavelength UV/HPLC with its attendant selectivity risk), or derivatise. Ninhydrin remains the most extensively validated and most frequently re-used derivatising agent for pregabalin, precisely because the chemistry is cheap, the reaction is selective for primary aliphatic amines (and therefore does not interfere with nortriptyline's secondary/tertiary amine or with methylcobalamin's corrin ring), and it has already been demonstrated to work in the presence of a co-formulated tricyclic antidepressant (Harkhani et al., 2023).

3.3 Pregabalin in Combination with Drugs Other than Nortriptyline and Vitamin B12

Pregabalin is co-formulated with a wide range of other actives for neuropathic and inflammatory pain, and the resulting method-development literature offers useful chromatographic precedents even though it does not address the specific triad under review. RP-HPLC methods have been validated for pregabalin with etoricoxib, using C8 or C18 columns with phosphate-buffer/acetonitrile or phosphate-buffer/methanol/acetonitrile mobile phases and UV detection between 200 and 240 nm, several of which are explicitly framed as stability-indicating and, in the most recent instances, benchmarked for 'greenness' using tools such as AGREE, AGREEprep, ComplexGAPI, and BAGI (Swathi, 2024). Similar RP-HPLC platforms have been developed for pregabalin with aceclofenac (Gelani et al., 2014; Pola & Sankar, 2014), with epalrestat, and, of greatest relevance to the vitamin B12 arm of this review, with methylcobalamin itself: Kannapan et al. (2009) describe an RP-HPLC method for pregabalin-methylcobalamin capsules using a water-methanol mobile phase with UV detection, and a further RP-HPLC method extends the pairing to a ternary combination with alpha-lipoic acid, another neuropathy adjunct, using a water-methanol-acetonitrile mobile phase with detection at 215 nm (Patel, N. D., et al., 2016). A UPLC method has also been reported for pregabalin with aceclofenac, and pregabalin-sildenafil combinations have been quantified by LC-MS/MS in animal pharmacokinetic studies.

4. Analytical Methods Reported for Nortriptyline

4.1 Individual Estimation of Nortriptyline

Because nortriptyline carries an intrinsic chromophore, its individual analytical literature is more conventional than pregabalin's, spanning direct UV-spectrophotometry, HPLC-UV, HPLC-fluorescence, electrochemical, and kinetic methods. Sherje and Dias (2022) reported an RP-HPLC method for nortriptyline hydrochloride in bulk and dosage form. Abhang et al. (2019) validated a UV-spectrophotometric area-under-curve (AUC) method over the 200-400 nm range in DMSO, linear from 5-25 micrograms/mL. Electrochemical approaches include a fast Fourier-transform continuous cyclic voltammetric method at a gold microelectrode, sensitive to sub-nanomolar concentrations (Norouzi et al., 2007), and a cathodic adsorptive stripping voltammetric method at bulk and pharmaceutical-formulation level (Jain & Radhapyari, 2008), reflecting the compound's electroactive tertiary-amine and ring system. Kinetic photometric and fluorimetric methods, resonance Rayleigh-scattering techniques, and quantum-dot-based spectrofluorimetric sensing methods have also been reported for nortriptyline in biological fluids, and HPLC with fluorescence detection has been used for plasma-level monitoring in psychiatric therapeutic drug monitoring.

4.2 Nortriptyline in Combination with Drugs Other than Pregabalin

Nortriptyline has most often been paired analytically with other central-nervous-system-active drugs. Ashour and Kattan (2012) developed an HPLC-UV method for nortriptyline with fluphenazine in low-dosage microgram quantities, and the same drug pair has separately been resolved by ion-selective PVC-membrane electrodes exploiting ion-pair complexation with sodium tetraphenylborate. Because nortriptyline and gabapentin are also co-prescribed for neuropathic pain, several RP-HPLC methods have been validated for this pairing, reporting retention times around 1.96 minutes (gabapentin) and 4.54 minutes (nortriptyline) with correlation coefficients above 0.999 for both analytes; several of these papers explicitly note in their introductions that, prior to their work, no chromatographic or spectroscopic method had been reported for the simultaneous estimation of gabapentin and nortriptyline. Nortriptyline has additionally been resolved from the closely related tricyclics amitriptyline and clomipramine, and from chlordiazepoxide, in single-run HPLC methods designed for multi-drug psychotropic panels.

5. Analytical Methods Reported for Vitamin B12 (Methylcobalamin/Cyanocobalamin)

5.1 Individual Estimation of Vitamin B12

Because methylcobalamin and cyanocobalamin both possess an intensely coloured, conjugated corrin-ring chromophore, UV-visible spectrophotometry is the most commonly reported single-analyte method, exploiting absorbance typically read between 265 and 361 nm in tablet dosage forms; one representative validated method reported accuracy of 98.46-100.67% and precision (RSD) below 1%. Because methylcobalamin is present in tablets only at microgram-level doses (frequently 500-1500 micrograms against 75-300 mg of pregabalin or nortriptyline), sensitivity and selectivity in the presence of large excesses of the co-formulated actives

is the dominant analytical challenge rather than detectability. RP-HPLC methods with UV or diode-array detection have been developed both for methylcobalamin alone and, more commonly, as part of stability-indicating multivitamin panels alongside thiamine, pyridoxine, nicotinamide, and folic acid, typically using gradient reversed-phase separations with ion-pairing reagents such as heptanesulphonic acid, and cyanocobalamin is frequently analysed on a separate, more retentive column (for example, a Suplex pKb-100 phase with methanol-water 22:78) because of its comparatively low tablet concentration relative to the other B-vitamins. For the most demanding matrices and lowest concentrations, HPLC coupled to inductively coupled plasma mass spectrometry (HPLC-ICP-MS), which quantifies the complexed cobalt atom rather than the organic chromophore, has been used by regulatory laboratories to resolve and confirm the different cobalamin species (cyano-, hydroxo-, adenosyl-, and methyl-cobalamin) in multivitamin products.

5.2 Vitamin B12 in Combination with Drugs Other than Pregabalin and Nortriptyline

Because methylcobalamin is a common neuropathy adjunct, it has been co-analysed with several drugs besides pregabalin and nortriptyline. UV-spectrophotometric and RP-HPLC methods have been validated for methylcobalamin with gabapentin, in one case using ninhydrin derivatisation of gabapentin itself (which, like pregabalin, is a GABA analogue lacking a strong chromophore) to allow simultaneous dual-wavelength measurement of the gabapentin-ninhydrin complex (405 nm) and methylcobalamin (351 nm) (Galande et al., 2010). Methylcobalamin has also been paired with dosulepin hydrochloride, another tricyclic antidepressant structurally analogous to nortriptyline, in a validated stability-indicating RP-HPLC method, and with epalrestat, an aldose-reductase inhibitor used for diabetic complications (Ponnekanti et al., 2021). This body of work is analytically instructive for the present triad because dosulepin and epalrestat are, chromatographically, close cousins of nortriptyline and pregabalin respectively, and the mobile-phase and detection strategies validated for these near-analogue pairs are directly transferable starting points.

6. Analytical Methods for the Pregabalin-Nortriptyline Binary Combination

The binary pregabalin-nortriptyline combination, marketed independently of the vitamin B12 triad for neuropathic pain, has attracted a comparatively larger and more mature body of analytical work, and is the closest existing precedent for the ternary method that remains to be developed.

Potluri et al. (2017) reported a validated, stability-indicating RP-HPLC method using a BDS C18 column (250 x 4.6 mm, 5 micron) with 0.1% perchloric acid-acetonitrile (55:45) mobile phase and UV detection at 210 nm, achieving linearity from 5-30 micrograms/mL for nortriptyline and 37.5-225 micrograms/mL for pregabalin, with recoveries of 100.6-101.65% and 100.59-101.74% respectively; forced-degradation studies confirmed adequate resolution of degradation products from both parent peaks. Because pregabalin has no native chromophore, this and comparable RP-HPLC methods necessarily rely on its very weak absorbance at 210 nm, which is workable for a binary tablet matrix but affords limited selectivity margin, and is one reason later workers turned to explicit derivatisation.

Patel (2020) took the derivatisation route directly, validating a UV-spectrophotometric Q-absorbance-ratio method (239 nm/235 nm, in methanol) for nortriptyline together with an RP-HPLC method, and, because pregabalin gave no usable signal at these wavelengths, converted it to its benzoylated derivative (read at 225 nm) using benzoyl chloride. Harkhani et al. (2023) instead used HPTLC with post-chromatographic ninhydrin derivatisation, developing bands on silica gel 60 F254 with an acetonitrile-methanol-triethylamine-water-formic acid mobile phase and achieving compact, well-resolved bands for nortriptyline (Rf 0.523) and derivatised pregabalin (Rf 0.279), with linearity of 50-300 ng/band and 350-2250 ng/band respectively and recoveries of 98.84-101.87%; the authors explicitly noted that no HPTLC method had previously been reported for this pair. An additional stability-indicating RP-HPLC method for the impurity profile of nortriptyline-pregabalin tablets has also been reported (Bagul et al., 2024), and further RP-HPLC and spectrophotometric variants for the pair exist in the regional pharmaceutical-sciences literature (Patel, Mewada, et al., 2017).

Collectively, the pregabalin-nortriptyline literature demonstrates that both non-derivatised, low-wavelength RP-HPLC and derivatisation-based UV/HPTLC strategies are workable for the binary pair, and that at least one HPTLC method has successfully combined ninhydrin derivatisation of pregabalin with direct measurement of nortriptyline in a single chromatographic run. This is the direct chemical precedent for extending the approach to a third, non-interfering analyte such as methylcobalamin.

7. Analytical Methods for the Pregabalin-Nortriptyline-Methylcobalamin (Vitamin B12) Ternary Combination

Despite the clinical prevalence of the triple fixed-dose combination, the literature specifically validating a method for all three actives together is sparse. Only one peer-reviewed, indexed method was identified with confidence: Sonulkar et al. (2023) developed and validated a UV-spectrophotometric simultaneous-equation method for pregabalin, methylcobalamin, and nortriptyline in sustained-release tablets, measuring absorbance at three selected wavelengths (219, 222, and 239 nm) in an ethanol-distilled water

solvent system. The method reported linear ranges of 30-150 micrograms/mL (pregabalin), 0.6-3.0 micrograms/mL (methylcobalamin), and 2-10 micrograms/mL (nortriptyline), with correlation coefficients at or above 0.99 for all three analytes at all three wavelengths, and recoveries within ICH-acceptable limits for all three drugs (99.30-101.61% pregabalin, 98.89-100.02% methylcobalamin, 99.69-100.23% nortriptyline).

Critically, this UV method does not derivatise pregabalin at all; it relies instead on multi-wavelength simultaneous-equation mathematics to resolve pregabalin's very weak native signal from the much stronger signals of nortriptyline and methylcobalamin at the same three wavelengths. While the reported validation data are within acceptance criteria, a method that depends on measuring pregabalin at wavelengths where it is not the dominant absorber inherently affords less specificity margin than a method in which pregabalin is first converted into an unambiguous, well-separated chromophore, particularly in the presence of tablet excipients, degradation products, or manufacturing impurities that may also absorb weakly in the low-UV region.

A second, chromatographic attempt at the ternary combination was also identified in the literature search: a comparative RP-HPLC/mixed-mode-chromatography study for nortriptyline, pregabalin, and methylcobalamin reporting robustness data across flow rate, organic-modifier percentage, and detection-wavelength variations. However, this work was published outside the mainstream indexed pharmaceutical- or analytical-chemistry journal literature, in a venue whose scope and editorial process are not oriented toward analytical chemistry, and its introduction and discussion sections show clear signs of generic, loosely referenced prose rather than the disciplined method-development narrative expected of a validated ICH-compliant assay. It is therefore noted here for completeness but is not treated as a robust precedent, and it does not, on inspection, describe any derivatisation step for pregabalin. No fully derivatisation-based, stability-indicating, chromatographic (RP-HPLC, UPLC, or HPTLC) method for the complete pregabalin-nortriptyline-methylcobalamin triad, published in an indexed, peer-reviewed pharmaceutical-analysis journal, was located during this review.

8. Research Gaps Identified

Synthesising the literature surveyed above, the following specific gaps emerge:

- Absence of a validated, stability-indicating chromatographic method for the ternary combination. Only one validated method (a UV-spectrophotometric simultaneous-equation approach) was identified for the complete pregabalin-nortriptyline-methylcobalamin triad in mainstream literature, and it is not chromatographic and not explicitly stability-indicating; no forced-degradation study establishing that pregabalin, nortriptyline, and methylcobalamin degradation products do not interfere with the parent-drug signals has been reported for the triad.

- No derivatisation-based method has yet been extended to the full triad. Ninhydrin, benzoylation, PDAB, and diazotisation-coupling derivatisation chemistries are all well validated for pregabalin alone and, in the case of ninhydrin and benzoylation, have already been successfully combined with nortriptyline in binary methods (Harkhani et al., 2023; Patel, 2020). None of these derivatisation strategies has yet been demonstrated to be compatible with methylcobalamin's corrin-ring chromophore in a single three-component assay, and the question of whether ninhydrin or other amine-selective reagents interfere with, or are masked by, methylcobalamin's strong visible-region absorbance has not been systematically investigated.

- Limited exploitation of HPTLC and modern UPLC platforms for the triad. HPTLC has been validated for the pregabalin-nortriptyline pair with ninhydrin derivatisation (Harkhani et al., 2023) and UPLC has been used for pregabalin with etoricoxib and aceclofenac, but neither platform has been reported for the ternary combination, despite HPTLC's particular suitability for low-cost multi-analyte screening in resource-limited quality-control settings.

- No green-chemistry or Analytical Quality-by-Design (AQbD) assessment for the triad. Several recent pregabalin-etoricoxib RP-HPLC methods have incorporated formal greenness metrics such as AGREE, AGREEprep, ComplexGAPI, and BAGI, and AQbD/experimental-design approaches have been used for pregabalin spectrophotometry alone and for unrelated multi-drug HPLC methods. No published method for pregabalin, nortriptyline, and methylcobalamin together has applied either a green-analytical-chemistry assessment or a formal AQbD/design-of-experiments optimisation strategy, despite both being increasingly expected by contemporary pharmaceutical-analysis journals.

- Sparse bioanalytical/pharmacokinetic coverage of the combination. While pregabalin (LC-MS/MS, GC-MS) and nortriptyline (HPLC-fluorescence) each have well-developed plasma-assay literatures individually, no LC-MS/MS method quantifying pregabalin, nortriptyline, and methylcobalamin simultaneously in a single biological-matrix run was identified, which would be of value for future pharmacokinetic or bioequivalence studies of the fixed-dose combination.

● Editorial/quality concerns around existing ternary reports. The one additional chromatographic attempt at the ternary combination identified during this search was published outside indexed pharmaceutical-analysis venues and lacked the methodological rigour expected for ICH-compliant validation, underscoring rather than filling the gap for a properly peer-reviewed, indexed, stability-indicating chromatographic method for this specific fixed-dose combination.

9. Scope for Future Method Development

The gaps identified above point toward a clear and well-precedented scope for new work. Building on the demonstrated compatibility of ninhydrin derivatisation with nortriptyline-containing matrices (Harkhani et al., 2023) and on the successful three-wavelength resolution of all three actives by simultaneous-equation UV spectrophotometry (Sonulkar et al., 2023), a logical next step is the development and full ICH Q2(R1) validation of a derivatisation-based, stability-indicating RP-HPLC or HPTLC method for pregabalin, nortriptyline, and methylcobalamin in combined tablet dosage form, with the following specific priorities:

● Systematic evaluation of ninhydrin, PDAB, benzoyl chloride, and diazotisation-coupling derivatisation of pregabalin in the presence of methylcobalamin, to establish which chemistry gives a derivative chromophore/wavelength sufficiently separated from methylcobalamin's strong native absorbance bands to allow unambiguous simultaneous quantitation without prior chromatographic separation, or, failing that, integration of the derivatisation step into a chromatographic (RP-HPLC/HPTLC) separation so that co-elution or spectral overlap with methylcobalamin is resolved by retention time or Rf rather than by wavelength alone.

● Forced-degradation (acid, base, oxidative, thermal, photolytic, and neutral hydrolysis) studies on the ternary mixture, extending the stability-indicating rigour already established for the pregabalin-nortriptyline pair (Potluri et al., 2017) to include methylcobalamin, which is itself photolabile and susceptible to degradation under the same stress conditions typically applied to the other two actives.

● Application of AQbD/design-of-experiments methodology (as already applied to single-analyte pregabalin spectrophotometry) to optimise derivatisation reaction time, temperature, and reagent concentration, and chromatographic mobile-phase composition, flow rate, and wavelength, for the ternary system, generating a documented design space rather than a one-factor-at-a-time optimisation.

● Formal green-analytical-chemistry assessment (AGREE, AGREEprep, ComplexGAPI, and/or BAGI scoring) of any newly developed method, in line with the trend already visible in the pregabalin-etoricoxib literature, to support adoption in quality-control laboratories under increasing environmental-sustainability expectations.

● Extension to HPTLC as a low-cost, high-throughput alternative to RP-HPLC for routine batch release testing, exploiting the same ninhydrin-derivatisation/selectivity logic already validated for pregabalin-nortriptyline HPTLC (Harkhani et al., 2023), with methylcobalamin resolved either by its distinctive pink/violet native colour on the plate or by densitometric scanning at a wavelength distinct from the derivatised pregabalin band.

● Bioanalytical extension via LC-MS/MS, building on existing individual pregabalin and nortriptyline plasma methods, to support future pharmacokinetic, bioequivalence, or therapeutic-drug-monitoring studies of the marketed fixed-dose combination, an area with essentially no published precedent for this specific triad.

A method developed along these lines would close a genuine and clinically relevant analytical gap: a validated, stability-indicating, appropriately derivatised chromatographic assay for a fixed-dose combination that is already in wide clinical use but that currently lacks a mainstream, indexed, chromatographic quality-control method addressing all three actives simultaneously.

10. Conclusion

Pregabalin, nortriptyline, and vitamin B12 (methylcobalamin) are rationally co-formulated for neuropathic pain, and each has an extensive individual and cross-combination analytical literature. Nortriptyline and methylcobalamin are both readily amenable to direct UV-visible and chromatographic detection because of their native chromophores, but pregabalin's aliphatic structure and absence of any chromophore or fluorophore has required the development of a diverse toolkit of derivatisation chemistries, of which ninhydrin derivatisation is the most extensively validated and most successfully transferred into combination methods. The pregabalin-nortriptyline binary combination is comparatively well served, including at least one validated HPTLC method employing ninhydrin derivatisation. The complete pregabalin-nortriptyline-methylcobalamin ternary combination, however, is supported by only one validated, indexed method, which is spectrophotometric rather than chromatographic and does not employ derivatisation. This represents a clear and actionable gap: a stability-indicating, derivatisation-based, chromatographic method for the ternary fixed-dose combination, ideally developed under an AQbD framework and benchmarked for green-analytical-chemistry

credentials, would meaningfully strengthen the quality-control and regulatory-science base for a combination already in widespread clinical use.

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