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RESEARCH ARTICLE

Isocratic Reversed-Phase HPLC Assay for the Simultaneous Estimation of Duloxetine Hydrochloride and Derivatized Pregabalin in Combined Pharmaceutical Formulations

Devi Sireesha Valluri, Sai Krishna N, Anjali A*, S S Prasanna Kumar Ponnaganti

Department of Pharmaceutical analysis, A K R G College of Pharmacy, Nallajerla, Andhra Pradesh, India

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Abstract: A validated isocratic reversed-phase high-performance liquid chromatography assay was developed for the simultaneous determination of duloxetine hydrochloride and pregabalin in combined pharmaceutical formulations. Due to the absence of a conjugated ultraviolet chromophore in the aliphatic backbone of pregabalin, pre-column derivatization via nucleophilic acylation using benzoyl chloride under alkaline conditions was implemented to incorporate a benzoyl moiety, enabling sensitive spectrophotometric detection. Separation was achieved on an Agilent 1100 Series HPLC system equipped with a reversed-phase stationary phase maintained at 25°C, utilizing an isocratic mobile phase of methanol, acetonitrile, and water in a 50:20:30 (v/v/v) ratio at a flow rate of 1.0 mL/min. Spectrophotometric detection was carried out at 241 nm for duloxetine and 208 nm for the derivatized pregabalin. Duloxetine and pregabalin eluted at retention times of 2.564 min and 6.321 min, respectively, exhibiting sharp peak symmetry and baseline resolution. The assay was validated in compliance with ICH Q2(R1) guidelines. Calibration plots exhibited linearity across 5–25 µg/mL for both compounds with correlation coefficients of 0.999. Repeatability and intermediate precision showed relative standard deviations below 1.0%. Mean analytical recoveries across 80%, 100%, and 120% spike levels ranged from 99.32% to 99.89% for duloxetine and 99.69% to 99.99% for pregabalin. The method showed ruggedness and robustness against minor variations in organic composition and wavelength, confirming its suitability for routine quality control testing of both active ingredients.

Keywords: Reversed-Phase HPLC; Duloxetine Hydrochloride; Pregabalin; Pre-column Derivatization; Method Validation.

1. Introduction

Duloxetine hydrochloride, chemically known as (+)-(S)-N-methyl-3-(naphthalen-1-yloxy)-3-(thiophen-2-yl)propan-1-amine hydrochloride, functions as a potent dual serotonin (5-HT) and norepinephrine (NE) reuptake inhibitor [1, 2]. It is extensively prescribed for managing major depressive disorders, generalized anxiety disorder, and chronic pain conditions, including diabetic peripheral neuropathy and fibromyalgia [1, 3]. Pregabalin, designated as (S)-3-(aminomethyl)-5-methylhexanoic acid, is a structural analogue of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) [1, 2]. Pregabalin acts as a selective ligand at the auxiliary alpha-2-delta subunit of voltage-gated calcium channels within the central nervous system, reducing calcium influx at nerve terminals and decreasing excitatory neurotransmitter release, which provides therapeutic relief in neuropathic pain, epilepsy, and fibromyalgia [1, 4].

Clinical protocols frequently combine duloxetine hydrochloride and pregabalin to achieve synergistic analgesic effects via complementary peripheral and central neurochemical mechanisms [1, 5]. Accurate and robust analytical procedures are required to ensure the concurrent quality, potency, and safety of these agents in fixed-dose co-formulations [1, 6].

Estimation of this combination poses a significant chromatographic challenge arising from divergent molecular structures and physicochemical properties [6, 7]. Duloxetine contains aromatic naphthalene and thiophene ring systems that confer strong ultraviolet absorption [2, 7]. Pregabalin is an aliphatic branched-chain amino acid derivative devoid of conjugated pi-electron networks or ultraviolet chromophores, resulting in negligible absorption above 200 nm [2, 8]. Direct spectrophotometric quantification of underivatized pregabalin in the low-ultraviolet region suffers from severe baseline drift, low absorptivity, and non-selective spectral interference from common formulation excipients [8, 9]. To overcome this detection barrier, pre-column derivatization targets the primary aliphatic amine moiety of pregabalin [8, 10]. Nucleophilic acylation using benzoyl chloride under Schotten-Baumann alkaline conditions readily attaches a conjugated benzoyl chromophore to the pregabalin backbone [8, 10]. This

* Corresponding author: Anjali A

structural modification shifts its absorption spectrum into a distinct ultraviolet zone, allowing simultaneous reversed-phase separation and spectrophotometric quantification alongside duloxetine [8, 11].

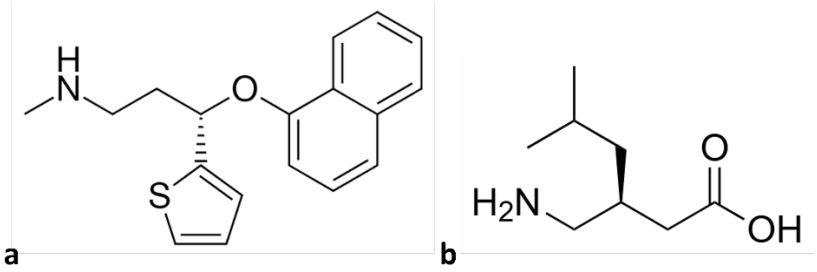

Figure 1. Structures of a. Duloxetine Hydrochloride and b. Pregabalin

Analytical methods reported for individual or combined determinations of duloxetine and pregabalin include ultraviolet-visible spectrophotometry, high-performance thin-layer chromatography, and high-performance liquid chromatography [3-6, 12]. However, several existing methods require ternary gradient programming, ion-pairing reagents with prolonged column re-equilibration periods, or complex tandem mass spectrometric detectors that are cost-prohibitive for high-throughput commercial batch release [11, 13]. The objective of this research was to develop and validate a rapid, sensitive, reproducible, and cost-effective isocratic reversed-phase liquid chromatographic assay coupled with pre-column benzoylation for the simultaneous estimation of duloxetine hydrochloride and pregabalin in bulk forms and combined pharmaceutical dosage units in full accordance with ICH Q2(R1) guidelines [14-17].

2. Methodology

2.1. Materials and Reagents

Reference standards of duloxetine hydrochloride and pregabalin were procured from Sunrise Remedies. HPLC-grade methanol, acetonitrile, and water were purchased from Finar Limited. Analytical reagent (AR) grade benzoyl chloride, sodium hydroxide, hydrochloric acid, and related analytical chemicals were obtained from Ranbaxy Chemicals. Membrane filters (0.45 µm nylon) were supplied by Millipore India.

2.2. Instrumentation and Chromatographic Conditions

Chromatographic separation was performed on an Agilent Technologies 1100 Series HPLC system equipped with a quaternary solvent delivery pump, an automated autosampler with a fixed 20 µL injection loop, a thermostatted column compartment, and a variable-wavelength ultraviolet-visible detector [14, 15]. System automation, signal acquisition, and chromatographic peak integration were processed using Agilent ChemStation software [15, 17].

Chromatographic separation was executed on a reversed-phase C18 analytical column (150 mm × 4.6 mm, 5 µm particle size) maintained at an ambient temperature of 25 ± 2°C [15, 17]. The optimized mobile phase consisted of methanol, acetonitrile, and HPLC-grade water mixed in an isocratic volumetric ratio of 50:20:30 (v/v/v) [15]. The mobile phase was vacuum-filtered through a 0.45 µm nylon membrane filter and ultrasonically degassed for 15 minutes prior to use [15]. The flow rate was set at 1.0 mL/min with an overall analytical run time of 8.0 minutes [15]. The sample injection volume was 20 µL, utilizing methanol as the sample diluent [15]. Detection wavelengths were monitored at 241 nm for duloxetine and 208 nm for the derivatized pregabalin analyte [15]. The fluidic path was equilibrated with the mobile phase for 30 minutes prior to analytical injections to secure a stable baseline [15].

2.3. Pre-Column Benzoylation Procedure

Pregabalin was derivatized via pre-column benzoylation based on nucleophilic displacement under alkaline conditions [8, 10]. An aliquot of pregabalin solution was combined with aqueous sodium hydroxide (0.1 M) to generate the unprotonated nucleophilic primary amine [8]. Benzoyl chloride solution was introduced dropwise and agitated, facilitating electrophilic attack at the carbonyl carbon to yield the N-benzoylated pregabalin derivative [8]. The reaction mixture was quenched and neutralized using dilute 5% hydrochloric acid, followed by dilution with methanol to target analytical concentrations [8].

2.4. Preparation of Standard Solutions

2.4.1. Preparation of Standard Stock Solutions

Primary standard stock solutions of duloxetine hydrochloride and pregabalin were prepared separately [15]. Precisely weighed quantities of 5.0 mg of duloxetine reference standard and 10.0 mg of pregabalin reference standard were transferred into independent 10 mL volumetric flasks [15]. Approximately 5 mL of HPLC-grade methanol was introduced into each flask, and the contents were sonicated for 10 minutes to achieve complete dissolution [15]. The flasks were brought to volume with methanol, producing primary stock concentrations of 500 µg/mL for duloxetine and 1000 µg/mL for pregabalin [15]. Stock solutions were protected from actinic light and stored at 4°C [15].

2.4.2. Preparation of Working Standard Solutions

Working calibration standards were prepared daily by transferring calculated aliquots of the primary stock solutions into 10 mL volumetric flasks, subjecting the pregabalin portion to the derivatization protocol, and adjusting to volume with methanol [15, 17]. A five-point calibration series containing both analytes simultaneously at concentrations of 5.0, 10.0, 15.0, 20.0, and 25.0 µg/mL was established [15]. Each working solution was filtered through a 0.45 µm syringe filter prior to instrumental injection [15].

2.5. Method Validation

The developed analytical procedure was validated for system suitability, specificity, linearity, sensitivity, precision, accuracy, robustness, and ruggedness in compliance with ICH Q2(R1) regulatory guidelines [14].

2.5.1. System Suitability and Specificity

System suitability was assessed by executing six replicate injections of a mixed working standard containing 10 µg/mL duloxetine and 20 µg/mL derivatized pregabalin [14, 15]. Retention time (t_r), resolution (R_s), theoretical plate count (N), and USP tailing factor (T) were evaluated against pharmacopeial specifications [14]. Specificity was determined by injecting methanol blank solutions, excipient placebo matrices, individual analyte standards, and mixed active drug preparations [14]. The absence of co-eluting peaks at the designated retention times confirmed that formulation additives did not compromise analyte quantification [14].

2.5.2. Linearity and Range

Linearity was established across five mixed concentration levels (5–25 µg/mL) for both analytes [14, 15]. Triplicate injections were performed at each concentration level [14]. Peak area responses were plotted against nominal concentrations, and statistical parameters including slope, y-intercept, and linear correlation coefficient (R^2) were determined via unweighted least-squares linear regression [14].

2.5.3. Precision and Sensitivity

Intra-day repeatability was evaluated through six consecutive injections of working standard concentrations (15 µg/mL duloxetine and 30 µg/mL pregabalin) [14, 15]. Intermediate (inter-day) precision was assessed by analyzing three independent concentration levels (10, 20, and 30 µg/mL) across three consecutive days [14]. The relative standard deviation (%RSD) was calculated to determine instrument repeatability and method precision [14]. Sensitivity was quantified through the Limit of Detection (LOD) and Limit of Quantitation (LOQ) using the standard deviation of the response (σ) and the slope of the calibration curve (S), according to:

$$LOD = \frac{3.3 \cdot \sigma}{S}$$

$$LOQ = \frac{10 \cdot \sigma}{S}$$

2.5.4. Accuracy, Robustness, and Assay of Formulations

Method accuracy was determined by the standard addition recovery technique [14, 16]. Known quantities of pure drug substances were spiked into synthetic placebo mixtures at 80%, 100%, and 120% of nominal assay levels, followed by extraction, derivatization, and triplicate analysis [14, 16]. Robustness was investigated by introducing deliberate minor deviations into mobile phase organic composition (methanol:acetonitrile:water ratios varied by ±1.0%) and detection wavelength (±1 nm) [14, 17]. Ruggedness was documented by executing the assay using a secondary analyst on a separate instrument [14, 17]. The optimized method was subsequently applied to determine active ingredient content in synthetic dosage blends [14, 16, 17].

3. Results and Discussion

3.1. Mechanism of Derivatization

Scanning methanolic standard solutions across the 200–400 nm spectral window showed maximum ultraviolet absorbance (λ_{max}) for duloxetine at 241 nm, attributed to $\pi \rightarrow \pi^*$ transitions within its conjugated naphthalene and thiophene moieties [7, 15]. Native pregabalin exhibited negligible absorbance above 210 nm, displaying an end-absorption fringe at 208 nm [8, 15].

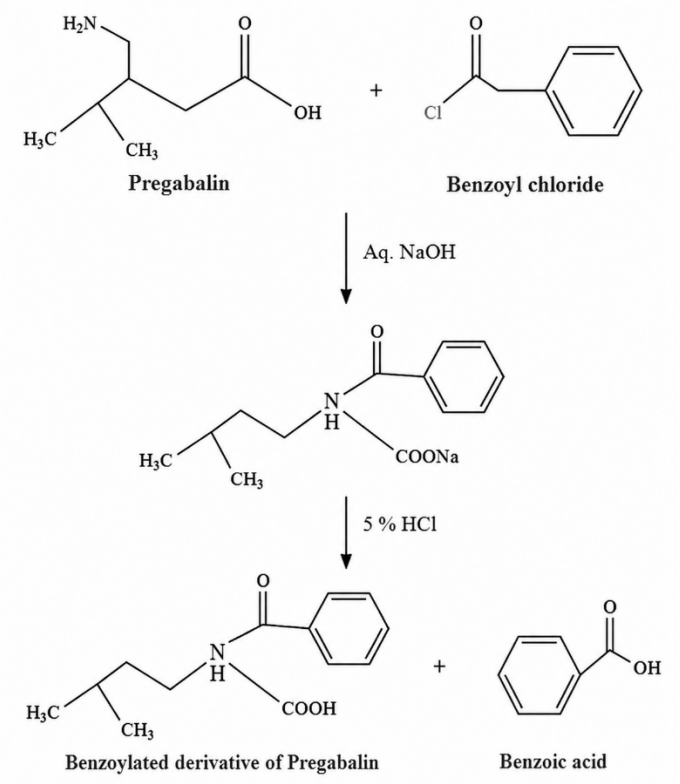

Figure 1. Reaction Mechanism for Pre-column Benzoylation of Pregabalin

Pre-column derivatization with benzoyl chloride under alkaline conditions covalently coupled a phenylcarbonyl chromophore to the primary amino group of pregabalin, yielding N-benzoyl pregabalin [8, 10]. This structural modification imparted high ultraviolet molar absorptivity, allowing sensitive detection at 208 nm alongside duloxetine [8, 15].

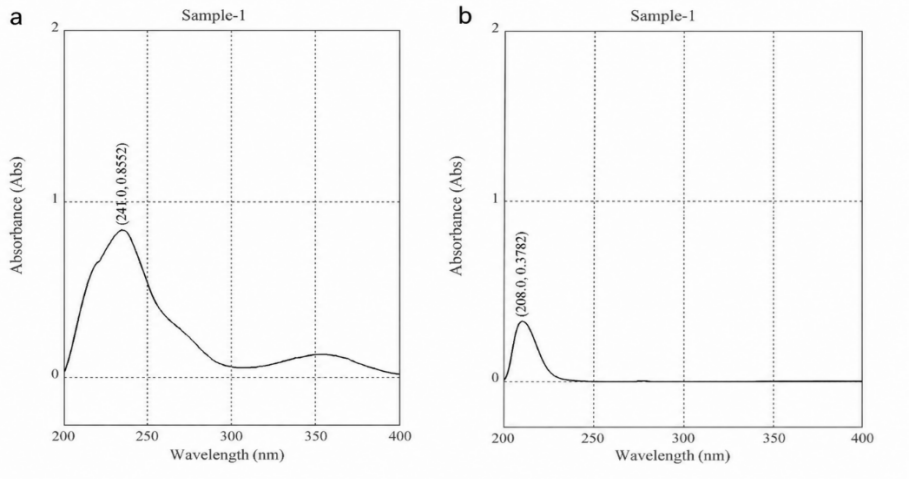

Figure 2. UV Absorption Spectra of Duloxetine and Pregabalin in Methanol

3.2. Chromatographic Separation and Mobile Phase Optimization

Mobile phase optimization prioritized symmetrical peak geometry, baseline resolution, and efficient elution within an 8-minute run time [15]. Mobile phase compositions evaluated at a constant flow rate of 1.0 mL/min are presented in Table 1 [15]. Initial screening with methanol:acetonitrile:water at 50:30:20 (v/v/v) produced broad peaks with poor baseline stability. Increasing methanol content to 70:10:20 (v/v/v) led to peak asymmetry and excessive band broadening. Formulations containing higher aqueous fractions (40:30:30 v/v/v) lengthened retention times beyond practical screening limits. Optimal separation was achieved with an isocratic ternary mixture of methanol, acetonitrile, and water at 50:20:30 (v/v/v), which produced sharp, symmetrical peaks with reproducible retention times of 2.564 min for duloxetine and 6.321 min for derivatized pregabalin [15].

Table 1. Optimization Trials for Mobile Phase Composition

Trial	Mobile Phase Ratio (Methanol:Acetonitrile: Water, v/v/v/)	Flow Rate (mL/min)	Injection Volume (µL)	Observation
1	50:30:20	1	20	Broad, poorly resolved peaks with slight tailing were detected.
2	70:10:20	1	20	Retention times lengthened as peaks became broader and symmetry diminished
3	60:20:20	1	20	Separation was only moderate; nevertheless, peak symmetry and resolution were unsatisfactory.
4	40:30:30	1	20	Peaks appeared well separated but exhibited extended retention times and minor baseline disturbances.
5 (Optimized)	50:20:30	1	20	Sharp, symmetric, and well-resolved peaks with a stable baseline and acceptable retention times were obtained.

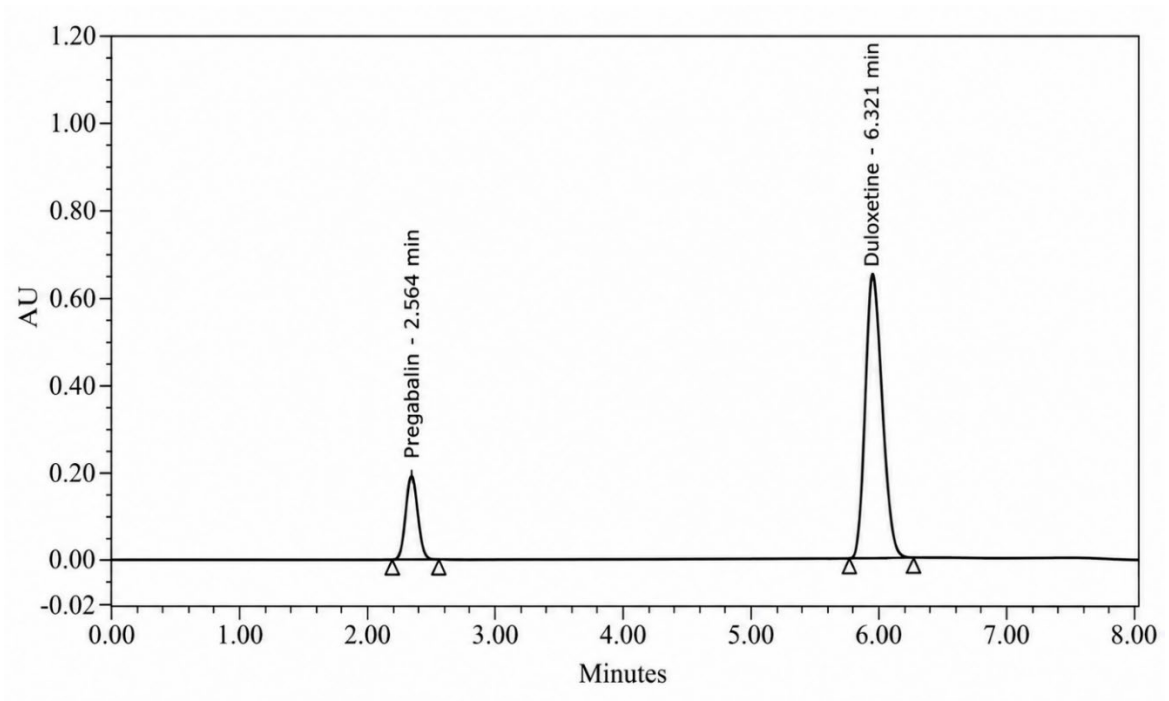

Figure 3. Optimized RP-HPLC Chromatogram of Duloxetine and Pregabalin
Duloxetine eluting at 2.564 min and derivatized pregabalin eluting at 6.321 min.

3.3. System Suitability and Specificity

System suitability tests confirmed the operational performance of the chromatographic system [14, 15]. Theoretical plate counts exceeded 7500 for duloxetine and 8300 for derivatized pregabalin, surpassing the pharmacopeial threshold of 2000 [14, 15]. USP tailing factors remained below 1.5, confirming peak symmetry [14, 15]. Chromatographic resolution between the analyte peaks was 2.37, satisfying the acceptance criterion ($R_s > 2.0$) [14, 15]. Retention time and peak area parameters exhibited high consistency

across replicate injections with %RSD values under 1.0% [14, 15]. No interfering peaks from diluent blanks or excipients were detected at analyte retention windows, confirming method specificity [14, 15].

Table 2. System Suitability Parameters for Duloxetine and Derivatized Pregabalin

Parameter	Duloxetine	Pregabalin	Acceptance criteria
Retention time (min)	2.090	5.289	—
% RSD (n=5)	0.67	0.82	NMT 2%
USP tailing factor	1.43	1.48	0.9–2.0
USP resolution	—	≥ 1.26	NLT 2
Theoretical plates	> 7500	> 8300	NLT 2000

3.4. Linearity, Sensitivity, and Precision

Linearity was verified over a concentration range of 5.0 to 25.0 µg/mL for both drugs [14, 15]. Linear regression analysis generated calibration equations of Y=41.231x+12.563 for duloxetine and Y=37.739x+3.2016 for pregabalin, each yielding a correlation coefficient (R^2) of 0.999 [14, 15].

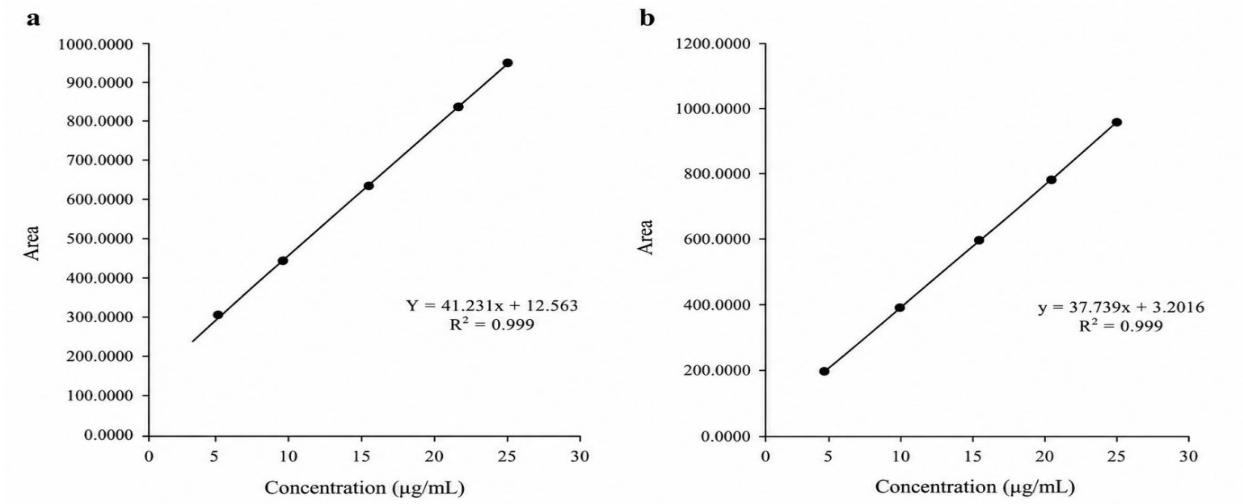

Figure 4. Calibration Curves of a. Duloxetine and b. Pregabalin

Sensitivity was confirmed by low detection and quantification limits [14, 15]. For duloxetine, LOD and LOQ were 0.654 µg/mL and 1.812 µg/mL, respectively. For derivatized pregabalin, LOD and LOQ were 0.368 µg/mL and 1.040 µg/mL, respectively.

Table 3. Results of Repeatability, Intra-day, and Inter-day precision

Compound	Parameter	Day 1 Mean Area	Day 2 Mean Area	Day 3 Mean Area	Overall Mean ± SD	Overall %RSD
Duloxetine HCl	Repeatability (15 µg/mL)	1178.45	—	—	1178.45 ± 2.11	0.179
	Intra-day (15 µg/mL)	1196.50	—	—	1196.50 ± 2.56	0.214
	Inter-day (10 µg/mL)	1056.70	1058.90	1054.30	1056.63 ± 2.30	0.218
	Inter-day (20 µg/mL)	1356.40	1359.10	1353.80	1356.43 ± 2.65	0.195
	Inter-day (30 µg/mL)	1592.30	1598.10	1587.20	1592.53 ± 5.45	0.342
Pregabalin	Repeatability (30 µg/mL)	2148.49	—	—	2148.49 ± 9.24	0.430
	Intra-day (30 µg/mL)	2184.73	—	—	2184.73 ± 13.54	0.620
	Inter-day (10 µg/mL)	2173.56	2181.20	2162.80	2172.52 ± 9.24	0.425
	Inter-day (20 µg/mL)	2188.45	2198.10	2175.40	2187.32 ± 11.39	0.521
	Inter-day (30 µg/mL)	2186.56	2195.40	2176.10	2186.02 ± 9.66	0.442

Precision studies confirmed high analytical repeatability and intermediate reproducibility [14, 15]. The intra-day repeatability assay produced %RSD values of 0.214% for duloxetine and 0.620% for pregabalin [14]. Inter-day precision evaluations across three concentrations over three consecutive days yielded %RSD values ranging from 0.216% to 0.362% for duloxetine and 0.480% to 0.670% for pregabalin, remaining well within the ICH acceptance limit of 2.0% [14].

3.5. Accuracy, Recovery, and Robustness

The accuracy of the method was validated via recovery studies across three spiking levels (80%, 100%, and 120%) [14, 16]. Analytical recoveries for duloxetine ranged from 99.32% to 99.89% with %RSD values between 0.41% and 0.50% [14]. Pregabalin recoveries ranged from 99.69% to 99.99% with %RSD values between 0.32% and 0.46% [14]. These values fall within the acceptable regulatory window of 98.0% to 102.0%, confirming the accuracy of the assay in the presence of excipients [14].

Table 4. Results of Recovery Studies

Analyte	Spiked Level (%)	Nominal Added (µg/mL)	Mean Found (µg/mL)	Mean Recovery (%)	SD	%RSD
Duloxetine HCl	80%	20.00	19.86	99.32	0.09	0.45
	100%	30.00	29.97	99.89	0.12	0.41
	120%	40.00	39.88	99.71	0.20	0.50
Pregabalin	80%	20.00	19.94	99.69	0.09	0.46
	100%	30.00	30.00	99.99	0.10	0.32
	120%	40.00	39.88	99.71	0.18	0.46

Robustness testing showed that minor alterations in mobile phase composition (±1.0% organic fraction) and detection wavelength (±1 nm) did not adversely affect resolution, theoretical plates, or peak symmetry [14, 17]. Across all varied chromatographic parameters, retention times remained stable within 2.107–2.315 min for duloxetine and 5.326–5.571 min for pregabalin, with %RSD values under 1.0% [14, 17].

Table 5. Method Robustness Under Deliberate Variations

Parameter	Variation	Drug	Retention Time (min)	Peak Area (mAU·s)	%RSD
Mobile phase	52:22:26 (v/v/v) (+2% Organic)	Duloxetine	2.213	1189.67	0.41
	52:22:26 (v/v/v) (+2% Organic)	Pregabalin	5.326	2183.77	0.36
	48:18:32 (v/v/v) (-2% Organic)	Duloxetine	2.107	1198.54	0.24
	48:18:32 (v/v/v) (-2% Organic)	Pregabalin	5.523	2210.07	0.19
Detection wavelength	-1 nm (240 nm / 207 nm)	Duloxetine	2.157	1056.27	0.27
	-1 nm (240 nm / 207 nm)	Pregabalin	5.571	1976.35	0.78
	+1 nm (242 nm / 209 nm)	Duloxetine	2.315	1201.78	0.19
	+1 nm (242 nm / 209 nm)	Pregabalin	5.543	2321.37	0.32

Inter-analyst ruggedness evaluation confirmed assay reproducibility, with mean label claim recoveries of 99.74% (%RSD = 0.34%) for duloxetine and 100.52% (%RSD = 0.25%) for pregabalin [14, 17].

Table 6. Results of Ruggedness Testing

Drug	Concentration (µg/mL)	Peak Area (Injection 1)	Peak Area (Injection 2)	Mean Peak Area	Amount Found (µg/mL)	% Label Claim	SD	%RSD
Duloxetine	15	1186.42	1192.18	1189.3	14.96	99.74	4.07	0.34
Pregabalin	30	2168.75	2176.43	2172.59	29.08	100.52	5.43	0.25

3.6. Formulation Assay

The validated method was applied to quantify duloxetine and pregabalin in synthetic combined formulations [16, 17]. The assay yielded active content determinations of 100.20% (15.03 µg/mL, %RSD = 0.34%) for duloxetine and 99.87% (29.98 µg/mL, %RSD = 0.21%) for pregabalin [14, 17]. These values fall within pharmacopeial acceptance criteria (98.0–102.0%), confirming the utility of the method for commercial release testing [14].

Table 7. Assay of the developed RP-HPLC Method for the Simultaneous Measurement of Pregabalin and Duloxetine in Pharmaceutical Dosage Forms.

Drug	Label Claim (µg/mL)	Peak Area (Injection 1)	Peak Area (Injection 2)	Mean Peak Area	Amount Found (µg/mL)	% Label Claim	SD	%RSD
Duloxetine	15	1187.42	1193.16	1190.29	15.03	100.2	4.06	0.34
Pregabalin	30	2171.84	2178.36	2175.1	29.98	99.87	4.61	0.21

4. Conclusion

An isocratic reversed-phase HPLC method coupled with pre-column benzoylation was developed and fully validated for the simultaneous determination of duloxetine hydrochloride and pregabalin. Pre-column nucleophilic acylation with benzoyl chloride overcame the poor ultraviolet absorption of native pregabalin, permitting reproducible dual-wavelength monitoring alongside duloxetine. Separation on a reversed-phase stationary phase with an isocratic mobile phase of methanol, acetonitrile, and water (50:20:30, v/v/v) achieved chromatographic resolution (R_s=2.37) and symmetrical peaks within an 8-minute run time. Method validation in accordance with ICH Q2(R1) showed high linearity, repeatability, intermediate precision, recovery, and robustness. The assay was applied to the quantification of both compounds in synthetic dosage mixtures without excipient interference. This analytical protocol provides an efficient, reproducible, and cost-effective method for routine commercial release, finished product release testing, and stability evaluation of combined duloxetine and pregabalin formulation.

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