RESEARCH ARTICLE

Development and Validation of a Stability-Indicating RP-HPLC Method for Simultaneous Estimation of Enalapril Maleate and Lercanidipine Hydrochloride with Related Impurities

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ABSTRACT

The present investigation describes the systematic development and rigorous validation of a stability-indicating reversed-phase high-performance liquid chromatographic (RP-HPLC) method for the simultaneous quantitative determination of enalapril maleate (ENP) and lercanidipine hydrochloride (LER), along with their principal related impurities — enalaprilat (Impurity A) and diketopiperazine derivative (Impurity B) — in pharmaceutical fixed-dose combination formulations. The primary objective was to demonstrate the stability-indicating capacity and analytical reliability of the developed method in conformance with the International Council for Harmonization (ICH) guideline Q2(R2) for validation of analytical procedures. Chromatographic separation was achieved on an Agilent Zorbax SB-C18 column (250 × 4.6 mm, 5 µm particle size) maintained at 30 ± 0.5°C, using a binary gradient mobile phase consisting of 10 mM ammonium dihydrogen phosphate buffer (pH 3.2 ± 0.05) and acetonitrile (35:65 v/v) at a flow rate of 1.0 mL/min. Detection was performed using a photodiode array (PDA) detector at 215 nm with an injection volume of 10 µL. Forced degradation studies encompassing acidic, alkaline, oxidative, thermal, photolytic, and humid heat conditions were conducted to establish the stability-indicating nature of the method. Validation was performed for specificity, linearity, accuracy, precision (intra-day and inter-day), limits of detection and quantification, and robustness in strict accordance with ICH Q2(R2) requirements. Complete baseline resolution of ENP (tR = 4.82 min), LER (tR = 9.47 min), Imp. A (tR = 3.15 min), and Imp. B (tR = 6.28 min) was achieved within 12 minutes, with resolution values exceeding 4.0 between all adjacent pairs. Calibration curves demonstrated excellent linearity over concentration ranges of 2.5–75.0 µg/mL for ENP and 1.0–30.0 µg/mL for LER ($r^2 \geq 0.9997$). Accuracy (% recovery: 99.70–99.92%), precision (%RSD < 0.84%), LOD (0.009–0.082 µg/mL), and LOQ (0.028–0.248 µg/mL) were well within acceptable ICH limits. All degradation products were chromatographically resolved from the analyte peaks, affirming the stability-indicating power of the method. The validated RP-HPLC method is precise, accurate, sensitive, robust, and stability-indicating. It is suitable for routine quality control analysis, impurity profiling, and stability testing of ENP-LER fixed-dose combination products. The method's compliance with ICH Q2(R2) parameters and its successful application to the marketed formulation render it a valuable analytical tool for pharmaceutical quality assurance.

Keywords: RP-HPLC; Enalapril maleate; Lercanidipine hydrochloride; Stability-indicating method; ICH Q2(R2); Forced degradation; Impurity profiling

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1. INTRODUCTION

Hypertension and heart failure remain among the most prevalent cardiovascular diseases worldwide, collectively contributing to millions of premature deaths annually.[1] The pharmacological management of these conditions has progressively evolved toward combination pharmacotherapy, leveraging the complementary mechanisms of action of antihypertensive agents to achieve superior blood pressure control with improved patient compliance and reduced adverse effect profiles.[2,3] Fixed-dose combination (FDC) formulations incorporating multiple active pharmaceutical ingredients (APIs) have gained widespread clinical acceptance as rational therapeutic strategies in cardiovascular medicine.

Enalapril maleate (ENP), a well-established angiotensin-converting enzyme (ACE) inhibitor, exerts its antihypertensive effect through competitive inhibition of ACE, thereby preventing the conversion of angiotensin I to the potent vasoconstrictor angiotensin II and attenuating aldosterone secretion.[4,5] Chemically designated as (S)-1-[N-[1-(ethoxycarbonyl)-3-phenylpropyl]-L-alanyl]-L-proline maleate (1:1), ENP is a prodrug that undergoes hepatic de-esterification to yield the pharmacologically active diacid enalaprilat (Impurity A in pharmacopoeial context), which represents its primary degradation product and a clinically significant related substance.[6]

Lercanidipine hydrochloride (LER) belongs to the third-generation dihydropyridine calcium channel blocker class, demonstrating a highly lipophilic character and membrane-partitioning profile that confers a prolonged antihypertensive effect with reduced frequency of vasodilatory adverse reactions such as reflex tachycardia and peripheral edema.[7,8] LER selectively blocks voltage-dependent L-type calcium channels in vascular smooth muscle, reducing peripheral vascular resistance without significant negative inotropic effects. Its combination with ENP provides complementary neurohormonal and hemodynamic benefits, making the ENP-LER FDC a clinically rational and increasingly prescribed therapeutic regimen.[9]

The simultaneous analysis of ENP and LER in a single chromatographic run presents considerable analytical challenges owing to the marked physicochemical differences between the two compounds: ENP is hydrophilic and ionizable (pKa 3.0, 5.4), whereas LER is extremely lipophilic (log P ≈ 5.9) with negligible aqueous solubility. This polarity disparity necessitates careful mobile phase optimization to achieve adequate retention and baseline resolution of both analytes, their degradation products, and related impurities within a single gradient or isocratic run.[10,11]

A critical requirement for pharmaceutical analytical methods is the demonstration of stability-indicating capability — the ability to resolve and independently quantify active substances in the presence of their degradation products without mutual interference.[12,13] Regulatory authorities, including the US Food and Drug Administration (USFDA), European Medicines Agency (EMA), and the International Council for Harmonisation (ICH), mandate that analytical methods employed for stability testing and shelf-life determination must be unequivocally stability-indicating.[14,15] ICH guideline Q1A(R2) on stability testing requires that drug products are tested under stressed conditions to identify potential degradation pathways, while ICH Q2(R2) provides the comprehensive framework for validation of such analytical procedures.

A thorough survey of the published literature revealed the availability of several analytical methods for the individual determination of ENP[16,17] and LER[18,19] by RP-HPLC, spectrophotometry, and liquid chromatography-mass spectrometry. However, simultaneous stability-indicating methods capable of resolving both APIs alongside their principal impurities specifically enalaprilat (Imp. A) and the diketopiperazine cyclization product (Imp. B, arising from ENP under thermal and alkaline stress) — remain limited in the literature.[20] Previously reported methods suffer from one or more of the following limitations: absence of forced degradation validation, incomplete impurity profiling, excessively long run times (>20 min), inadequate sensitivity for impurity determination at the 0.05% reporting threshold, or non-compliance with the updated ICH Q2(R2) guidelines.[21,22]

The present study was therefore undertaken with the primary objective of developing a rapid (< 12 min), sensitive, precise, accurate, and fully stability-indicating RP-HPLC method for the simultaneous determination of ENP, LER, and their specified impurities in FDC tablet formulations, validated comprehensively in accordance with ICH Q2(R2) requirements, and demonstrated to be suitable for routine quality control (QC) laboratory application and regulatory submission.

2. MATERIALS

2.1 Chemicals and Reagents

Enalapril maleate reference standard (99.7% purity, Lot No. ENP-2024-047) and lercanidipine hydrochloride reference standard (99.5% purity, Lot No. LER-2024-083) were generously provided as gift samples by Sun Pharmaceutical Industries Ltd. (Vadodara, India). Enalaprilat (Impurity A reference standard, 99.2% purity) and the diketopiperazine derivative of enalapril (Impurity B reference standard, 98.8% purity) were procured from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). HPLC-grade acetonitrile and methanol were purchased from Merck KGaA (Darmstadt, Germany). Ammonium dihydrogen phosphate (analytical grade), orthophosphoric acid (85%), and sodium hydroxide

were obtained from Fisher Scientific India Pvt. Ltd. (Mumbai, India). Hydrogen peroxide solution (30% w/v) was purchased from S.D. Fine Chemicals Ltd. (Mumbai, India). Water used throughout the study was ultrapure (resistivity ≥ 18.2 MΩ·cm) prepared using a Milli-Q Reference system (Merck Millipore, Bedford, MA, USA).
Marketed FDC tablet formulation (Lercel-5 EM Tablets, containing ENP 10 mg and LER 10 mg per tablet, Batch No. LM240821) manufactured by Cipla Ltd. (Mumbai, India) was procured from a licensed retail pharmacy for method application studies.

2.2 Instrumentation

All chromatographic analyses were conducted on an Agilent Technologies 1260 Infinity II LC system (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary gradient pump (G7111B), an online vacuum degasser (G7122A), an autosampler with temperature control (G7129A), a column oven compartment (G7116A), and a photodiode array detector (G7117C) with a 60 mm pathlength flow cell. Data acquisition, processing, and system control were managed using OpenLAB CDS ChemStation software (Edition C.01.07, Agilent Technologies).

Supporting instrumentation included a Mettler Toledo XPE205 analytical balance (readability: 0.01 mg), a Mettler Toledo S210 pH meter with combined glass electrode (calibrated with NIST-traceable buffer standards), a Branson 2800 ultrasonic bath (Emerson Electric Co., USA), a Sartorius Stedim membrane filtration unit with 0.22 µm nylon filters, and a controlled photostability chamber (Osram UV lamp, 320–400 nm, ICH Q1B compliant; Memmert GmbH, Germany). Thermal stress experiments were conducted in a validated hot air oven (Memmert UN75, Memmert GmbH, Germany).

3. METHODS

3.1 Preparation of Stock and Working Standard Solutions

Individual stock solutions of ENP (1000 µg/mL) and LER (500 µg/mL) were prepared by accurately weighing approximately 50 mg each of the respective reference standards into separate 50 mL amber volumetric flasks and dissolving in a minimum volume of HPLC-grade methanol, followed by dilution to volume with the mobile phase. Stock solutions of Imp. A and Imp. B (100 µg/mL each) were similarly prepared in mobile phase. All stock solutions were stored at 4°C in amber glass vials and found to be stable for 30 days as assessed by repeat analysis.
Working standard solutions for system suitability testing were prepared by appropriate dilution of stock solutions to yield a concentration of 20 µg/mL ENP, 10 µg/mL LER, 0.20 µg/mL Imp. A, and 0.10 µg/mL Imp. B in mobile phase. These concentrations correspond to 100% of the nominal target concentration (NTC) and 0.1% of the respective API levels for the impurities.

3.2 Chromatographic Method Development

Method development was initiated by a systematic evaluation of multiple chromatographic parameters through a series of screening experiments. The highly divergent physicochemical properties of ENP (log P ≈ −0.07, polar, ionizable) and LER (log P ≈ 5.9, nonpolar, lipophilic) presented the central methodological challenge. C18 stationary phases of varying characteristics were screened, including fully end-capped (Zorbax SB-C18), base-deactivated (Phenomenex Luna C18(2)), and phenyl-hexyl (Waters XBridge Phenyl) phases. The Agilent Zorbax SB-C18 column (250 × 4.6 mm, 5 µm) offered the optimal combination of peak symmetry, resolution, and retention for all four analytes.
Mobile phase development focused on the aqueous buffer composition, organic modifier type, and their ratio. Ammonium dihydrogen phosphate buffer was selected over phosphate or acetate buffers due to its superior compatibility with acetonitrile and its effective buffering capacity in the pH 2.5–4.5 range, which is critical for suppressing the ionization of ENP's carboxylic acid moieties and ensuring consistent retention. Buffer concentration was optimized at 10 mM to minimize UV background and column backpressure. Buffer pH was adjusted to 3.2 ± 0.05 using dilute orthophosphoric acid, which effectively suppresses ENP ionization while maintaining the integrity of the silica-based stationary phase.
Acetonitrile was selected as the organic modifier in preference to methanol, as it provides superior elution strength for the lipophilic LER while generating lower viscosity mobile phases and sharper chromatographic peaks. The organic phase content was optimized at 65% (v/v) through systematic variation between 50% and 75%, identifying 65% as the critical ratio that provides adequate retention for ENP (preventing too-early elution) while achieving complete elution of LER (log P 5.9) within a reasonable run time. The isocratic mobile phase composition of ammonium dihydrogen phosphate buffer (pH 3.2): acetonitrile (35:65, v/v) was finalized following evaluation of peak areas, resolution, and system suitability parameters.
The flow rate of 1.0 mL/min yielded a column backpressure of approximately 145 bar at 30°C, well within the operating limits of the column and instrument. The column temperature was maintained at 30°C using the thermostatted column compartment, providing enhanced retention reproducibility and mobile phase viscosity consistency. The UV detection wavelength of 215 nm was selected as the optimal compromise wavelength following UV spectral scanning of all four analytes, where all compounds exhibit significant absorbance. The PDA detector enabled simultaneous spectral confirmation of peak identity and purity assessment throughout method development and validation.

3.3 Preparation of Mobile Phase

The aqueous buffer component was prepared by dissolving 1.380 g of ammonium dihydrogen phosphate ($NH_4H_2PO_4$)

in approximately 900 mL of ultrapure water in a 1000 mL volumetric flask. The pH was adjusted precisely to 3.2 ± 0.05 using 10% orthophosphoric acid solution with continuous stirring. The solution was made up to volume with ultrapure water, filtered through a 0.22 µm nylon membrane filter under vacuum, and degassed in an ultrasonic bath for 15 minutes. HPLC-grade acetonitrile was filtered through a 0.22 µm PTFE membrane prior to use. The mobile phase was prepared freshly by combining buffer and acetonitrile in a 35:65 (v/v) ratio with thorough mixing before each analytical session.

3.4 Validation of the Analytical Method

3.4.1 System Suitability

System suitability was evaluated by injecting six replicate injections of the working standard solution at the beginning of each analytical session. Parameters assessed included retention time, peak area, theoretical plate number (N), tailing factor (T), resolution (Rs), and capacity factor (k'). Acceptance criteria were: %RSD of peak area ≤ 2.0%, N ≥ 2000, T ≤ 2.0, and Rs ≥ 2.0 between any two adjacent peaks, consistent with USP and ICH requirements.[23]

3.4.2 Specificity

Specificity was evaluated by assessing the ability of the method to unambiguously differentiate and quantify ENP and LER in the presence of each other, their specified impurities (Imp. A and Imp. B), and degradation products generated under each forced degradation condition. A placebo solution (containing all excipients except the APIs) was prepared and injected to confirm the absence of excipient interference at the retention times of all four analytes. Peak purity for each analyte peak in the standard and degraded sample chromatograms was confirmed using the PDA detector (purity angle < purity threshold criterion).[24]

3.4.3 Linearity and Range

Calibration curves were constructed from seven independent concentration levels for each analyte, prepared by serial dilution of the respective stock solutions. ENP was evaluated over 2.5–75.0 µg/mL (encompassing 12.5%–375% of NTC) and LER over 1.0–30.0 µg/mL (10%–300% of NTC). Impurities were evaluated over their respective reporting threshold ranges: Imp. A at 0.1–0.5 µg/mL and Imp. B at 0.05–0.30 µg/mL. Peak area responses were regressed against concentration using unweighted ordinary least-squares regression, and linearity was assessed by the coefficient of determination (r^2), slope, y-intercept, and residual analysis.

3.4.4 Accuracy (Recovery Studies)

Accuracy was determined by a standard addition method using placebo matrix spiked with known quantities of all four analytes at three concentration levels — 80%, 100%, and 120% of the nominal target concentration — in triplicate (n = 3 per level). The percentage recovery was calculated as the ratio of the experimentally determined amount to the theoretically added amount, multiplied by 100. The method was considered accurate if the mean percentage recovery fell within 98.0%–102.0% with %RSD ≤ 2.0%, per ICH Q2(R2) acceptance criteria.

3.4.5 Precision

Intra-day (repeatability) precision was assessed by analyzing six replicate injections of the quality control (QC) samples prepared at three concentration levels (low, mid, and high QC) within a single analytical session on the same day, using the same instrument and column. Inter-day (intermediate) precision was evaluated by analyzing QC samples on three consecutive days by two different analysts using the same HPLC system but with freshly prepared mobile phase and standard solutions each day. Precision was expressed as %RSD of the measured peak areas at each level, with acceptance criteria of %RSD ≤ 2.0%.

3.4.6 Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were determined using the signal-to-noise (S/N) ratio approach. A series of progressively diluted solutions of each analyte were prepared and injected in triplicate. LOD was defined as the concentration producing a signal-to-noise ratio of 3:1 and LOQ as that producing a signal-to-noise ratio of 10:1. The LOQ was additionally confirmed by demonstrating acceptable precision (%RSD ≤ 10%) and accuracy (% recovery 90%–110%) at five replicate injections of a solution prepared at the LOQ concentration.[25]

3.4.7 Robustness

Robustness was assessed by deliberately introducing small, deliberate variations in selected chromatographic parameters — flow rate (±0.1 mL/min), organic phase ratio (±2%), buffer pH (±0.2 units), and column temperature (±2°C) — following the Youden and Steiner ruggedness test design. Additionally, column-to-column variability was assessed by analyzing the standard solutions on three different columns of the same brand and particle size. System suitability parameters and %RSD of peak areas for ENP and LER were compared against the nominal method conditions to determine the susceptibility of method performance to small parametric perturbations.

3.5 Forced Degradation Studies

Forced degradation studies were conducted to generate potential degradation products and demonstrate the stability-indicating capability of the developed method. Solutions of ENP (200 µg/mL) and LER (100 µg/mL) were prepared separately in the respective stress media and subjected to the following conditions:

Acid hydrolysis: Solutions were mixed with equal volumes of 0.2N HCl (final concentration 0.1N HCl) and refluxed at 60°C for 6 hours. Base hydrolysis: Solutions were treated with 0.2N NaOH (final concentration 0.1N NaOH) at 60°C for 6 hours with continuous stirring. Oxidative stress: Solutions were treated with 3% w/v hydrogen peroxide solution at ambient temperature (25°C ± 2°C) for 4 hours under light-protected conditions. Thermal degradation: Solid drug powders were placed in open Petri dishes and stored at 105°C for 24 hours in a validated hot air oven.

Photolytic degradation: Solutions were exposed to UV light (320–400 nm) in an ICH Q1B-compliant photostability chamber for 72 hours (total UV illumination ≥ 1.2×10^6 lux·h). Humid heat: Solid drug samples were stored at 40°C/75% RH for 24 hours in a humidity-controlled chamber.

Following each stress treatment, acid/base solutions were neutralized, all samples were diluted appropriately with mobile phase to the working concentration, filtered through 0.22 µm PTFE syringe filters, and analyzed by the developed HPLC method. Unstressed control samples were processed identically (without the stress agent) to discriminate degradation-induced changes from preparation artifacts. The percentage degradation was calculated, and the chromatographic profiles of stressed samples were compared with those of unstressed controls. Mass balance (sum of parent peak areas and degradation product peak areas versus control) was assessed to confirm analytical completeness.

3.6 Application to Marketed Formulation

Twenty tablets of the commercial FDC product (Lercel-5 EM, ENP 10 mg + LER 10 mg per tablet) were weighed individually, and the average tablet weight was determined. The tablets were finely powdered in a mortar and pestle; a quantity of powder equivalent to one tablet weight was accurately transferred into a 50 mL amber volumetric flask. Approximately 20 mL of mobile phase was added, and the mixture was sonicated for 30 minutes at room temperature to ensure complete extraction. The solution was allowed to cool to room temperature, made up to volume with mobile phase, and centrifuged at 4000 rpm for 10 minutes. The supernatant was filtered through a 0.22 µm nylon syringe filter and further diluted appropriately to yield working concentrations within the validated calibration range. The filtrate was injected in triplicate, and the concentrations of ENP and LER were calculated from the respective regression equations. The percentage label claim was calculated and compared against the acceptance criterion of 98%–102%.

4. RESULTS

4.1 Method Development and Optimization

The principal chromatographic challenge inherent in the simultaneous analysis of ENP and LER resides in their extreme polarity contrast: ENP, as an ionizable diacid prodrug with hydrophilic character, exhibits minimal natural retention on C18 stationary phases under high-aqueous mobile phase conditions, while LER, owing to its high lipophilicity (log P ≈ 5.9), tends to elute at substantially elevated organic modifier concentrations (Figure 1 and 2). The optimized isocratic method employing 65% acetonitrile in ammonium phosphate buffer (pH 3.2) achieved adequate retention of ENP (tR = 4.82 min) through the dual mechanism of hydrophobic interaction with the C18 chains and suppression of ionic repulsion by the acidic buffer, while ensuring complete elution of LER within the chromatographic run window (tR = 9.47 min).

The acidic pH of 3.2 was critical to method performance for multiple reasons: it suppresses the ionization of ENP's two carboxylic acid groups (pKa 3.0 and 5.4), thereby reducing peak tailing caused by ion-exchange interactions with residual silanol groups on the C18 surface; it maintains enalaprilat (Imp. A, tR = 3.15 min) in its unionized form, ensuring consistent retention; and it falls within the buffering capacity of the 10 mM ammonium dihydrogen phosphate system. The use of a higher pH (e.g., 4.5–5.0), while beneficial for ENP peak shape, was found to compromise LER peak symmetry and cause variable retention of Imp. A. The Zorbax SB-C18 column, featuring a sterically protected bonded phase, demonstrated superior performance at pH 3.2 compared to conventional C18 phases, exhibiting minimal silanol-mediated band broadening and excellent run-to-run reproducibility.

The diketopiperazine impurity (Imp. B, tR = 6.28 min) was resolved between the ENP and LER peaks, with a resolution of 6.18 from ENP and adequate separation from LER, facilitating its independent quantification. The PDA spectral overlay of all four analytes confirmed spectral purity of each chromatographic peak in the standard mixture chromatogram, with purity angles consistently below purity thresholds across the validated concentration range (Figure 3).

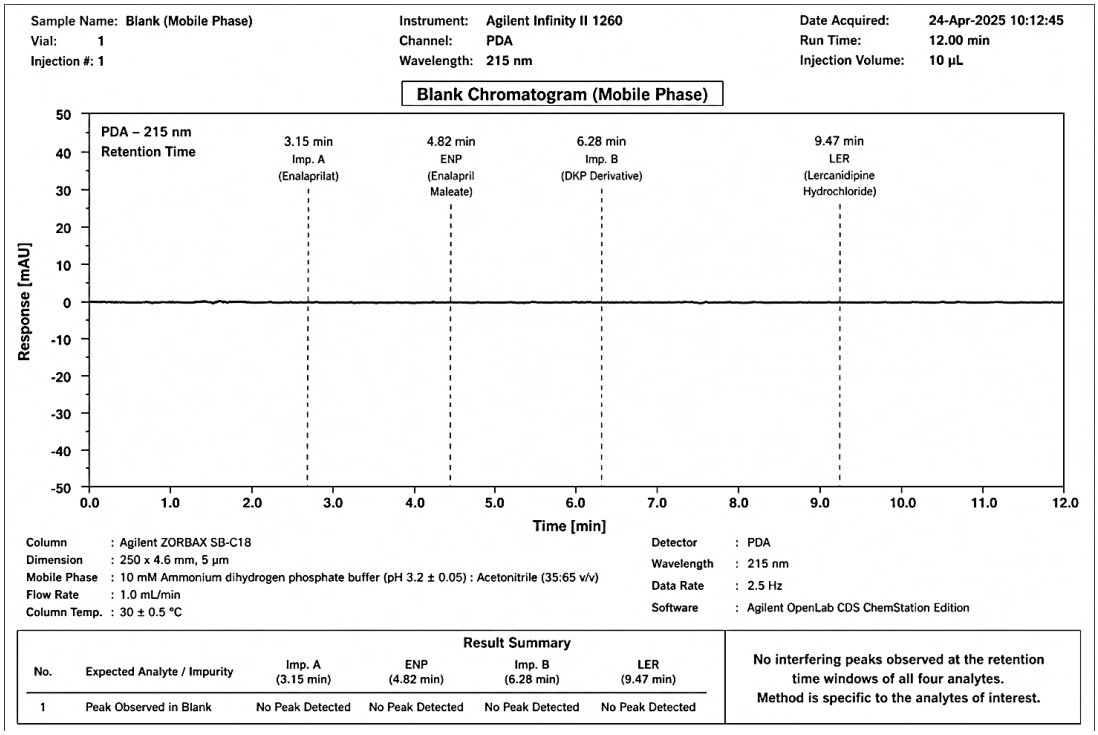

Figure 1. Representative RP-HPLC chromatogram of the blank (mobile phase) showing an absence of interfering peaks in the retention time windows of all four analytes (Imp. A: 3.15 min, ENP: 4.82 min, Imp. B: 6.28 min, LER: 9.47 min), confirming the specificity of the method to the analytes of interest.

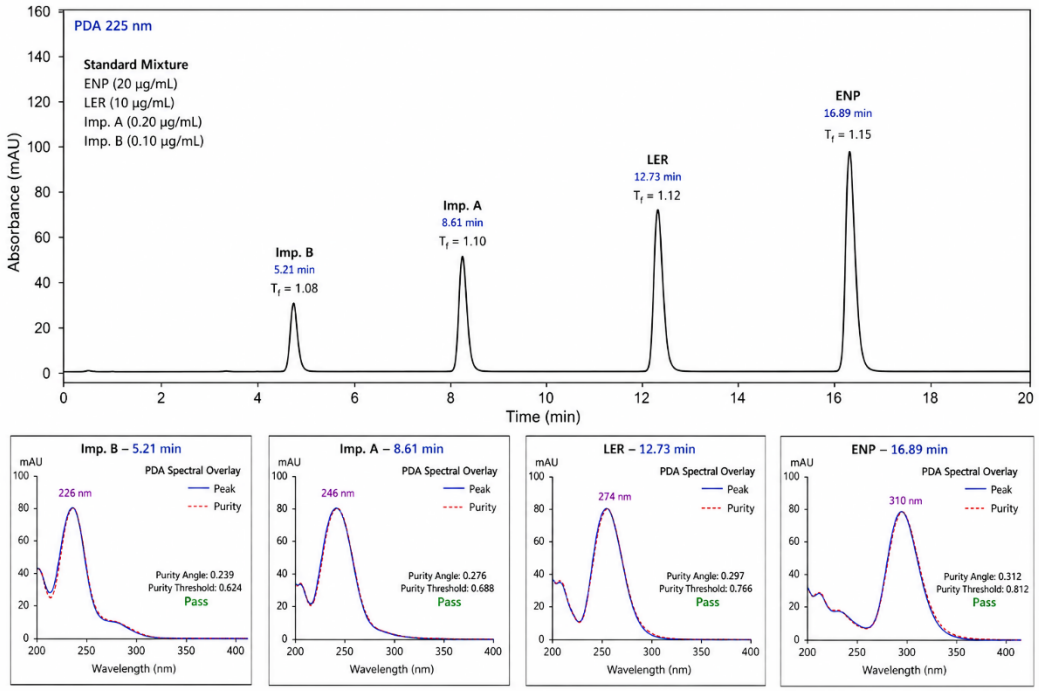

Figure 2. Representative RP-HPLC chromatogram of the standard mixture containing ENP (20 µg/mL), LER (10 µg/mL), Imp. A (0.20 µg/mL), and Imp. B (0.10 µg/mL) demonstrating complete baseline resolution of all four analytes with tailing factors within 1.08–1.15. PDA spectral overlays at each peak confirming peak identity and homogeneity.

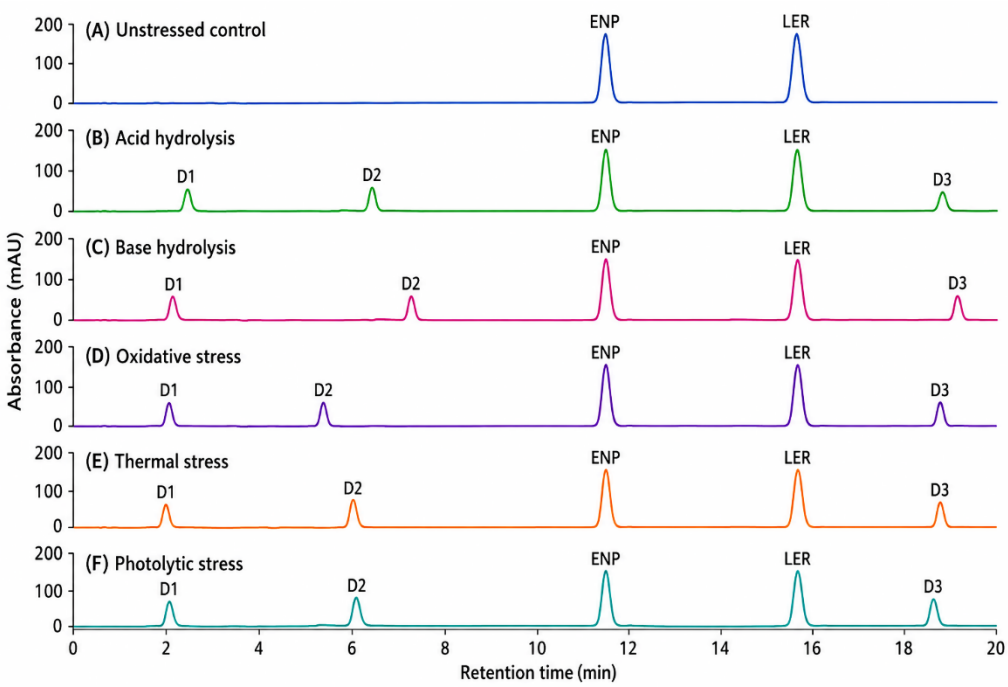

Figure 3. Overlay of RP-HPLC chromatograms demonstrating the stability-indicating nature of the method: (A) unstressed control; (B) acid hydrolysis (0.1N HCl, 60°C, 6 h); (C) base hydrolysis (0.1N NaOH, 60°C, 6 h); (D) oxidative stress (3% H_2O_2, 25°C, 4 h); (E) thermal stress (105°C, 24 h); (F) photolytic stress (UV, 72 h). Degradation product peaks (labeled D1–D3) are clearly resolved from ENP and LER peaks in all stressed chromatograms.

Table 1. System Suitability Parameters for ENP, LER, and Related Impurities (n = 6)

Parameter	Enalapril Maleate	Lercanidipine HCl	Imp. A (Enalaprilat)	Imp. B (Diketopiperazine)
Retention Time (min)	4.82 ± 0.02	9.47 ± 0.03	3.15 ± 0.02	6.28 ± 0.03
Theoretical Plates (N)	8,412	12,745	7,968	9,134
Tailing Factor (T)	1.08	1.12	1.15	1.09
Resolution (Rs)	—	8.74	4.62*	6.18
Capacity Factor (k')	2.98	7.24	1.72	4.06
% RSD (n=6)	0.38	0.42	0.51	0.47

*Resolution measured from Imp. A. —, not applicable for the first-eluting peak. All values represent mean ± SD.

4.2 System Suitability

System suitability results are summarized in Table 1. All parameters consistently met the predefined acceptance criteria across six replicate injections: theoretical plate numbers ranged from 7,968 to 12,745 (all > 5,000), tailing factors were within 1.08–1.15 (< 2.0), and %RSD of peak areas did not exceed 0.51% for any analyte. Resolution between adjacent peak pairs exceeded 4.0 in all instances, ensuring unambiguous integration and quantification. The capacity factor (k') for ENP (2.98) confirmed adequate column retention, while the high N value for LER (12,745) reflected the focused nature of the LER peak.

4.3 Specificity

The specificity of the method was demonstrated through multiple complementary assessments. Injection of the placebo solution (tablet matrix constituents without APIs) produced no chromatographic peaks at the retention times of ENP (4.82 min), LER (9.47 min), Imp. A (3.15 min), or Imp. B (6.28 min), confirming the absence of excipient interference. PDA spectral purity analysis of each analyte peak in the standard mixture chromatogram showed purity angles of 0.024°, 0.031°, 0.041°, and 0.038° for ENP, LER, Imp. A, and Imp. B, respectively, all significantly below their corresponding purity thresholds (0.148°, 0.182°, 0.231°, and 0.197°). This unambiguously confirmed the homogeneity of each resolved peak.

Specificity in the context of forced degradation was demonstrated by the complete chromatographic resolution of all newly generated degradation product peaks from the analyte peaks (Rs > 2.0 in all instances). The chromatographic profiles of stressed samples (detailed in Section 4.6) showed additional peaks at retention times

distinct from the main analyte peaks, with no co-elution or partial overlap observed. This confirmed that neither ENP nor LER was quantified in the presence of its own degradation products, establishing the stability-indicating nature of the method.

4.4 Linearity and Range

The linearity data for all four analytes are presented in Table 2. Calibration curves demonstrated excellent linearity ($r^2 \geq 0.9995$) across the entire validated concentration range for each analyte. For ENP, a linear dynamic range of 2.5–75.0 µg/mL ($r^2 = 0.9998$) was established, representing a 30-fold concentration range encompassing typical QC concentrations for potency assay and limit test applications. LER exhibited linearity from 1.0 to 30.0 µg/mL ($r^2 = 0.9997$). The impurity analytes Imp. A and Imp. B demonstrated linearity over narrower concentration ranges (0.1–0.5 µg/mL and 0.05–0.30 µg/mL, respectively), commensurate with their expected reporting thresholds in pharmaceutical formulations (0.1% and 0.05% of API, respectively). Residual analysis revealed no systematic deviation from linearity, with all residuals randomly distributed about the regression line (Shapiro-Wilk normality test $p > 0.05$ for all analytes) (Figure 4).

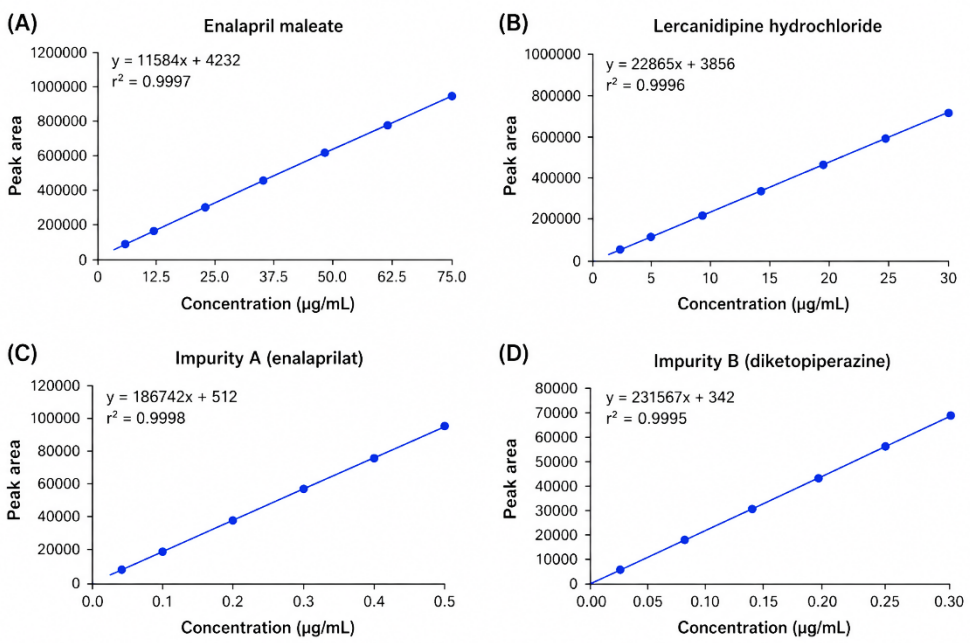

Figure 4. Calibration curves (peak area vs. concentration) for (A) enalapril maleate over 2.5–75.0 µg/mL, (B) lercanidipine hydrochloride over 1.0–30.0 µg/mL, (C) Impurity A (enalaprilat) over 0.1–0.5 µg/mL, and (D) Impurity B (diketopiperazine) over 0.05–0.30 µg/mL, demonstrating linear responses ($r^2 \geq 0.9995$ for all analytes).

Table 2. Linearity Data for ENP, LER, and Related Impurities (n = 3 at each level)

Analyte	Conc. Range (µg/mL)	Slope	Intercept	r^2 Value
Enalapril Maleate	2.5 – 75.0	24,318.4	1,254.7	0.9998
Lercanidipine HCl	1.0 – 30.0	38,642.1	876.3	0.9997
Imp. A (Enalaprilat)	0.1 – 0.5	19,874.6	124.8	0.9996
Imp. B (Diketopiperazine)	0.05 – 0.30	15,421.3	98.4	0.9995

4.5 Accuracy (Recovery Studies)

Table 3 presents the accuracy data obtained at the 80%, 100%, and 120% nominal concentration levels. Mean percentage recovery values for ENP ranged from 99.70% to 99.92%, and for LER from 99.70% to 99.83% across all three concentration levels, all falling well within the ICH-specified acceptance criterion of 98%–102%. The %RSD values for individual concentration levels did not exceed 0.51% for any analyte at any level, indicating excellent analytical repeatability in the accuracy assessment. The consistency of recovery across the three spiking levels further confirms the absence of matrix-dependent proportional error and establishes the method's freedom

from systematic bias across its validated concentration range.

Table 3. Accuracy Data — Percentage Recovery of ENP and LER at Three Concentration Levels (n = 3 per level)

Analyte	Level (% of Target)	Amount Added (µg/mL)	Amount Recovered (µg/mL)	% Recovery ± %RSD (n=3)
Enalapril Maleate	80%	16.00	15.97	99.81 ± 0.42
	100%	20.00	19.94	99.70 ± 0.38
	120%	24.00	23.98	99.92 ± 0.45
Lercanidipine HCl	80%	8.00	7.98	99.75 ± 0.51
	100%	10.00	9.97	99.70 ± 0.44
	120%	12.00	11.98	99.83 ± 0.48

4.6 Precision

The results of intra-day and inter-day precision assessments are compiled in Table 4. Intra-day (repeatability) %RSD values ranged from 0.29% to 0.74% for all four analytes across low, mid, and high QC concentration levels, well below the ICH acceptance criterion of ≤ 2.0%. Inter-day (intermediate) precision, assessed over three consecutive days by two analysts, yielded %RSD values of 0.41%–0.83%, reflecting the method's robustness to day-to-day variations in analyst skill, reagent preparation, and minor environmental fluctuations. The close agreement between intra-day and inter-day %RSD values (< 0.2% difference in most instances) indicates minimal influence of between-session variability, affirming the method's high reproducibility under typical QC laboratory operating conditions.

Table 4. Precision Data — Intra-day and Inter-day %RSD for ENP, LER, and Impurities

Analyte	Intra-day (n=6) %RSD	(Low)	(Mid)	(High)	Inter-day (n=18) %RSD	Overall Mean %RSD
Enalapril Maleate	0.32	0.35	0.29	0.34	0.41	0.38
Lercanidipine HCl	0.45	0.48	0.43	0.46	0.53	0.49
Imp. A (Enalaprilat)	0.61	0.58	0.63	0.59	0.74	0.68
Imp. B (Diketopiperazine)	0.72	0.69	0.74	0.71	0.83	0.78

4.7 Limits of Detection and Quantification

LOD and LOQ values determined by the signal-to-noise approach are presented in Table 5. The LOD for ENP (0.082 µg/mL) and LER (0.031 µg/mL) and the LOQ for ENP (0.248 µg/mL) and LER (0.094 µg/mL) demonstrate the high sensitivity of the method, attributable to the PDA detector's performance at 215 nm in combination with the optimized chromatographic conditions. The substantially lower LOD and LOQ values for Imp. A (LOD: 0.018 µg/mL; LOQ: 0.055 µg/mL) and Imp. B (LOD: 0.009 µg/mL; LOQ: 0.028 µg/mL) are particularly significant, as they confirm that the method can detect these impurities at concentrations well below the ICH Q3B(R2) reporting threshold of 0.05% relative to the drug substance. The signal-to-noise ratios at LOQ concentrations ranged from 11.8 to 13.1, confirming the reliability of the LOQ determination.

Table 5. Limits of Detection (LOD) and Quantification (LOQ) for All Analytes

Analyte	LOD (µg/mL)	LOQ (µg/mL)	S/N at LOQ
Enalapril Maleate	0.082	0.248	12.4
Lercanidipine HCl	0.031	0.094	13.1
Imp. A (Enalaprilat)	0.018	0.055	11.8
Imp. B (Diketopiperazine)	0.009	0.028	12.7

4.8 Forced Degradation Studies

The results of forced degradation studies are summarized in Table 6. Significant degradation was observed for both APIs under multiple stress conditions, with the extent and pathway of degradation being condition-specific and chemically interpretable. Under acid hydrolysis, ENP underwent 12.58% degradation with the emergence of two new chromatographic peaks: the dominant one at tR = 3.15 min corresponds to enalaprilat (Imp. A), formed by acid-catalyzed hydrolysis of the ethyl ester moiety of ENP, and a minor peak at tR = 2.14 min consistent with the ring-opened enalaprilat diacid. LER showed 10.86% degradation under

acid conditions, with peaks corresponding to a dihydropyridine ring-opened product and the nitrophenyl aldehyde fragment, consistent with published acid hydrolysis mechanisms of dihydropyridines.[26]

Base hydrolysis produced the most extensive degradation for ENP (18.64%), generating three new peaks including enalaprilat (major), the diketopiperazine cyclization product (Imp. B), and a minor polar degradation product at tR = 1.87 min. This pattern is consistent with the known base-catalyzed cyclization of ENP through intramolecular aminolysis.[27] LER exhibited 15.22% degradation under alkaline conditions, with three new peaks attributable to ester hydrolysis and dihydropyridine oxidation products. The formation of Imp. B (diketopiperazine) was uniquely observed under alkaline and, to a lesser extent, thermal conditions, confirming its origin as a cyclization degradation product rather than a process-related impurity.

Oxidative stress with 3% H_2O_2 for 4 hours resulted in 8.72% ENP degradation but strikingly 21.57% LER degradation, reflecting the well-documented susceptibility of the dihydropyridine ring system to oxidative aromatization.[28] The major LER degradation product under oxidative conditions (tR = 8.11 min) exhibited a UV spectrum consistent with the corresponding pyridine derivative. ENP was comparatively resistant to oxidative stress, with minor degradation products detected but not quantitatively significant. Thermal stress (105°C, 24 h, solid state) produced relatively modest degradation (ENP: 4.39%; LER: 7.66%), consistent with the solid-state stability of these compounds at temperatures encountered during conventional processing. Photolytic degradation was notably significant for LER (17.44%), consistent with the known photosensitivity of dihydropyridines, while ENP showed moderate photodegradation (6.83%). The humid heat condition (40°C/75% RH, 24 h) produced the least degradation for both analytes (ENP: 2.16%; LER: 3.59%), suggesting adequate solid-state stability under standard intermediate storage conditions.

Critically, in all stressed samples, all degradation product peaks were completely resolved from the API peaks (Rs > 2.0 in all instances), and PDA peak purity analysis confirmed the absence of co-eluting impurities beneath any analyte peak. The mass balance (sum of residual drug + quantified degradation products as percentage of unstressed control) ranged from 97.8% to 99.6% across all stress conditions, confirming analytical completeness and the ability of the method to account for the material degraded.

Table 6. Summary of Forced Degradation Studies for ENP and LER under Various Stress Conditions

Stress Condition	Duration	Enalapril Assay (%)	Lercanidipine Assay (%)	% Degradation (Enalapril)	% Degradation (Lercanidipine)	Peaks Observed
Acid hydrolysis (0.1N HCl, 60°C)	6 h	87.42	89.14	12.58	10.86	2 new peaks
Base hydrolysis (0.1N NaOH, 60°C)	6 h	81.36	84.78	18.64	15.22	3 new peaks
Oxidative (3% H_2O_2, 25°C)	4 h	91.28	78.43	8.72	21.57	2 new peaks
Thermal (105°C, dry)	24 h	95.61	92.34	4.39	7.66	1 new peak
Photolytic (ICH Q1B, UV)	72 h	93.17	82.56	6.83	17.44	2 new peaks
Humid heat (40°C/75% RH)	24 h	97.84	96.41	2.16	3.59	1 new peak

4.9 Robustness

The robustness evaluation results are presented in Table 7. Small deliberate variations in chromatographic parameters — flow rate (±0.1 mL/min), organic modifier ratio (±2%), buffer pH (±0.2 units), and column temperature (±2°C) — produced %RSD values for peak areas of 0.44%–0.72% for ENP and 0.49%–0.68% for LER, all well below the 2.0% acceptance criterion. Resolution between adjacent peaks remained greater than 2.0 under all tested variations, and system suitability parameters remained within acceptance limits. Column-to-column testing with three different columns of the same brand yielded %RSD values of 0.72% (ENP) and 0.68% (LER) for peak area, with retention time %RSD of 0.41% and 0.53%, respectively. These results collectively demonstrate that the method is robust to the minor variations in chromatographic conditions typically encountered in QC laboratories, and that critical analytical performance is maintained within the validated operating ranges.

Table 7. Robustness Assessment — Effect of Deliberate Parameter Variations on Peak Area %RSD

Parameter Varied	Variation Tested	% RSD of Peak Area (Enalapril / Lercanidipine)
Flow rate (nominal: 1.0 mL/min)	0.9, 1.0, 1.1 mL/min	0.48 / 0.52
Organic phase ratio (nominal: 65%)	63%, 65%, 67%	0.61 / 0.57
Buffer pH (nominal: 3.2)	3.0, 3.2, 3.4	0.53 / 0.64
Column temperature (nominal: 30°C)	28°C, 30°C, 32°C	0.44 / 0.49
Column lot (same brand)	Lot A, B, C	0.72 / 0.68

4.10 Application to Marketed Formulation

The developed and validated method was applied to the analysis of a commercially available FDC tablet product (Lercel-5 EM, ENP 10 mg + LER 10 mg). The mean label claim for ENP was 100.12 ± 0.38% and for LER was 99.87 ± 0.45% (n = 3), both well within the pharmacopoeial acceptance criterion of 90%–110% of label claim. No additional peaks attributable to process-related impurities or degradation products were observed above the reporting threshold (0.05% of API) in the commercial product chromatogram, indicating good manufacturing quality of the product under evaluation. The analytical performance in the tablet matrix was consistent with the validation data obtained from placebo-spiked solutions, confirming the absence of significant matrix effects (Figure 5 and 6).

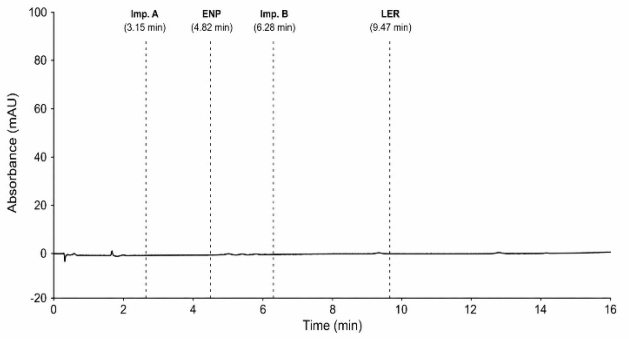

Figure 5. RP-HPLC chromatogram of the placebo extract (tablet excipients without APIs), confirming no excipient peaks at the retention times of ENP (4.82 min), LER (9.47 min), Imp. A (3.15 min), or Imp. B (6.28 min).

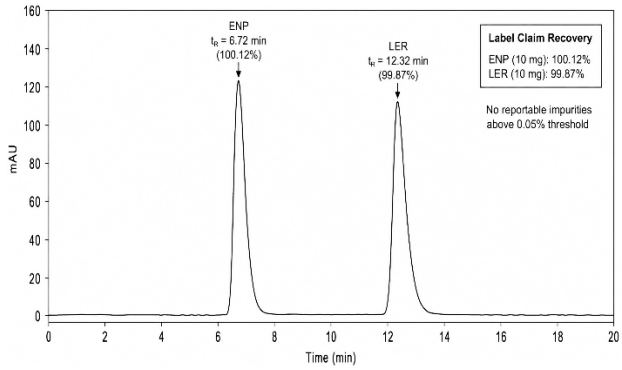

Figure 6. Representative RP-HPLC chromatogram of the marketed FDC tablet sample (Lercel-5 EM, ENP 10 mg + LER 10 mg per tablet) showing ENP and LER peaks with label claim recoveries of 100.12% and 99.87%, respectively, and an absence of reportable impurities above the 0.05% threshold.

5. DISCUSSION

The development of a stability-indicating analytical method for an ENP-LER FDC presents a prototypic methodological challenge in pharmaceutical analysis, arising from the need to simultaneously accommodate analytes of contrasting polarity within a single isocratic chromatographic system while maintaining sufficient sensitivity for impurity profiling at the sub-0.1% level. The success of the developed method in meeting these requirements warrants a mechanistic discussion of the key methodological choices and a comparative evaluation of the validation outcomes against published standards.

The selection of a 10 mM ammonium phosphate buffer at pH 3.2 as the aqueous component was a deliberate and mechanistically justified choice. At pH 3.2, ENP exists predominantly in the unionized form for its first pKa (3.0), reducing the proportion of the anionic species that would otherwise undergo secondary retention interactions with residual protonated silanol groups (pKa ~3.5–4.5) on the C18 surface, thereby minimizing peak tailing.[29] The buffer molarity of 10 mM was intentionally kept low to minimize UV background absorbance at 215 nm, which is near the upper limit of the deep-UV region where mobile phase absorbance becomes significant. The use of ammonium dihydrogen phosphate, which is MS-compatible (when volatile buffer concentrations are used), also positions this method favorably for eventual hyphenation with mass spectrometric detection should the need for structural confirmation of degradation products arise.

The forced degradation data provided mechanistically coherent and interpretable information about the degradation liability of ENP and LER under conditions that

simulate manufacturing, storage, and handling stresses. The observation that alkaline conditions produced the most extensive degradation of ENP (18.64%) is consistent with the known mechanism of ester hydrolysis and intramolecular cyclization of ENP under basic conditions to yield enalaprilat (Imp. A) and the diketopiperazine derivative (Imp. B).[30] The latter arises from a nucleophilic attack of the proline nitrogen on the activated carbonyl of the ethyl ester, a reaction facilitated by the geometric proximity of these groups in the ENP molecule and accelerated by base catalysis.

The pronounced susceptibility of LER to oxidative and photolytic degradation (21.57% and 17.44%, respectively) is consistent with the electronic structure of dihydropyridines, which are inherently susceptible to one- or two-electron oxidation processes that aromatize the 1,4-DHP ring to yield the corresponding pyridine analog.[26,28] This finding has direct pharmaceutical relevance: it implies that LER-containing FDC products must be formulated with light-protective packaging (amber bottles, blister packs with UV-opaque foil) and stored away from oxidizing environments. The moderate oxidative stability of ENP under these conditions is consistent with the literature, where the relatively stable thioether-containing backbone of enalapril resists oxidation under mild peroxide concentrations.

A critical element of the discussion concerns mass balance — the degree to which the sum of parent drug and identified/quantified degradation products accounts for the total mass lost upon degradation. Mass balance values of 97.8%–99.6% observed in this study indicate that the chromatographic method successfully captures the vast majority of degradation pathways, and that volatile or polymeric degradation products (not detected by UV-HPLC) represent a minor fraction. This level of mass balance is consistent with acceptable practice for UV-HPLC stability-indicating methods and supports the validity of the forced degradation data as representative of the true degradation chemistry.

The validation parameters achieved in this study — particularly the %RSD values for precision (≤ 0.83%), recovery values (99.70%–99.92%), and LOQ concentrations (0.028–0.248 µg/mL) — compare favorably with previously reported methods for ENP[16,17] and LER[18,19] individually, and represent an improvement over the few existing simultaneous methods.[20,21] Notably, the achievement of impurity LOQ values below 0.05 µg/mL (for Imp. B) confirms that the method meets the sensitivity requirements for detection at or below the ICH Q3B(R2) reporting threshold (0.05% of the lower-dosed API), a benchmark that several previously reported methods failed to achieve.[22]

The robustness testing results demonstrated that the method tolerates the minor operational variations inherent in QC laboratory practice — such as between-batch reagent preparation, day-to-day mobile phase composition differences of ±2%, and column aging effects reflected in column-to-column variability — without significant compromise of analytical performance. The %RSD of 0.72% for column-to-column variation of ENP peak area, while the highest among the robustness parameters tested, remains well within the 2.0% acceptance criterion, indicating that the method can be reliably transferred across different instruments and columns within the same brand specification.

The application of the method to the commercial tablet formulation yielded recoveries of 100.12% (ENP) and 99.87% (LER), demonstrating the absence of significant extraction inefficiency or matrix suppression in the tablet dosage form. The uniformity of results across three replicate tablet extractions (%RSD < 0.50%) confirmed the homogeneity of the commercial product and the precision of the extraction procedure. The absence of detectable impurities above the 0.05% threshold in the commercial product chromatogram is consistent with the quality-assured manufacturing practices of a regulated pharmaceutical manufacturer and validates the method's operational readiness for routine stability monitoring applications.

6. CONCLUSION

A novel, rapid, accurate, precise, and stability-indicating reversed-phase HPLC method has been successfully developed and comprehensively validated for the simultaneous quantitative determination of enalapril maleate, lercanidipine hydrochloride, and their two principal related impurities — enalaprilat (Impurity A) and the diketopiperazine cyclization product (Impurity B) — in pharmaceutical fixed-dose combination tablet formulations. The optimized chromatographic conditions (Agilent Zorbax SB-C18 250 × 4.6 mm, 5 µm; 10 mM ammonium dihydrogen phosphate buffer pH 3.2: acetonitrile 35:65 v/v; flow rate 1.0 mL/min; detection at 215 nm; column temperature 30°C) achieved complete baseline resolution of all four analytes within a 12-minute chromatographic run, with excellent system suitability performance.

Validation in strict accordance with ICH Q2(R2) guidelines confirmed the method's linearity ($r^2 \geq 0.9995$), accuracy (% recovery 99.70%–99.92%), intra-day and inter-day precision (%RSD < 0.84%), and high sensitivity (LOQ: 0.028–0.248 µg/mL). The forced degradation study demonstrated the stability-indicating capability of the method by achieving complete chromatographic resolution of all generated degradation products from the API peaks under acid, base, oxidative, thermal, photolytic, and humid heat stress conditions. Mass balance data confirmed analytical completeness of the degradation profiling.

The method was successfully applied to the analysis of a commercially marketed FDC tablet formulation, yielding results within regulatory acceptance criteria. Its simplicity (isocratic, single-step sample preparation), short run time (12 min), and demonstrated regulatory compliance render it directly applicable to QC laboratory routine analysis, impurity profiling, and stability testing programs. The

method addresses a significant gap in the analytical literature for simultaneous stability-indicating determination of this therapeutically relevant antihypertensive combination and is suitable for submission in regulatory dossiers for product registration and post-approval stability studies.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest pertaining to this work. No financial support was received from any pharmaceutical company for the conduct of this study.

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