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ESTIMATION OF PERINDOPRIL ARGININE, INDAPAMIDE AND AMLODIPINE IN BULK AND FIXED DOSE COMBINATION USING STABILITY INDICATING REVERSE PHASE HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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Keywords:

Perindopril arginine, Indapamide, Amlodipine, Reverse phase ion-pair liquid chromatography, Stability indicating assay method, Fixed-dose combination, Validation (ICH Q2 R1)

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ABSTRACT: A simple, accurate, and precise stability-indicating reverse phase high pressure liquid chromatographic method was developed and validated for the estimation of Perindopril arginine, Indapamide, and Amlodipine in the bulk and pharmaceutical dosage form. Chromatographic separation was carried out on ZORBAX RX C8 (250 mm × 4.6 mm, 5 µm) as a stationary phase with a mobile phase of gradient system of Phosphate Buffer with pH 2 containing Decane Sulphonate as ion-pairing agent (A) and Acetonitrile (B) at detection wavelength 215 nm with flow rate 1.0 mL/min at column temperature 40 ºC. The t_R of Perindopril, Indapamide, and Amlodipine was 31.39 ± 0.21 min, 25.73 ± 0.41 min, and 36.95 ± 0.47 min, respectively. The method was linear over the concentration ranges 50-150 µg/ mL for Perindopril, 12.5-37.5 µg/ mL for Indapamide and 50-150 µg/ mL for Amlodipine. The LOD was 0.99 µg/ mL for Perindopril, 0.87 µg/ mL for Indapamide and 3.99 µg/ mL for Amlodipine. The LOQ was 3.02 µg/ mL for Perindopril, 2.6 µg/ mL for Indapamide and 12.08 µg/ mL for Amlodipine. Under forced degradation conditions, Perindopril degraded significantly under acidic, alkaline, oxidative, and thermal stress conditions degraded moderately under photolytic stress conditions and degraded the least under neutral conditions. Indapamide degraded significantly under acidic, alkaline, and oxidative stress conditions; degraded moderately under the neutral condition and degraded the least under thermal and photolytic stress conditions. Amlodipine degraded significantly under acidic, alkaline and oxidative stress conditions, degraded moderately under photolytic stress conditions and showed negligible degradation under neutral conditions.

INTRODUCTION: A fixed-dose combination of Perindopril Arginine (PER), Indapamide (IND), and Amlodipine (AML) (10 + 2.5 + 10 mg) is a substitution therapy for the treatment of essential hypertension in patients already controlled with perindopril / Indapamide fixed-dose combination and amlodipine, taken at the same dose level.

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Perindopril (PER), chemically, (2S, 3AS, 7AS–1 – [(2S)–2 -{[(2S)-1-ethoxy-1- oxapentan-2yl] amino} propanoyl] – 2, 3, 3a, 4, 5, 6, 7, 7a – octa-hydroindole – 2 – carboxylic acid} **Fig. 1** is official in BP [1], USP [2], EP [3] as erbumine salt form.

It is a lipophilic drug with a long duration of action, is characterized by high tissue affinity for angiotensin-converting enzyme (ACE), and inhibits the formation of angiotensin II [4]. It has shown high efficacy in patients with hypertension, stable coronary artery disease, heart failure, diabetes mellitus, and cerebrovascular disease. Some methods have been described determination of PER, either alone or in combination, including

spectrophotometry [5, 6], LC with tandem MS [7, 8], HPLC [9, 10,] and HPTLC [11-12]. Indapamide (IND) is chemically 4-chloro – N–[(2RS)–2–methyl –2, 3–dihydro –1H –indol –1-yl] – 3 – sulfamoyl-benzamide **Fig. 2** .It is official in BP [13], USP [14], EP [15]. It is considered as a thiazide-like diuretic, as shown by changes in the level of sodium, potassium, urea, and uric acid in plasma and a decrease in body weight, but these changes are lower when compared with thiazides. These results would suggest that IND has both diuretic and vasodilator properties [16]. Reported methods have been described for the determination of IND, either alone or in combination, including spectrophotometry [17-18], HPLC [19-20], HPTLC [21, 22] and LC – MS/MS [23, 24]. Amlodipine {AML; 3-ethyl-5-methyl (4RS)-2-[(2- aminoethoxy) methyl] -4- (2-chlorophenyl) -6- methyl -1,4 – dihydropyridine – 3,5- dicarboxy-latesulfonate} is official in BP [25], USP [26], IP [27] and EP [28] as benzene sulphonic acid salt form **Fig. 3**. It is one of the calcium channel blockers which induces nitrous oxide release from coronary microvessels through a kinin–dependent mechanism and contribute positively to the therapeutic action of ACE inhibitors [29]. A literature survey revealed many methods for estimation of AML, either alone or in combination, *via* spectrophotometry [30, 31], HPLC [32, 33], HPTLC [34, 35,] and LC-MS/MS [36, 37, 38].

The aim of the present work was to develop a stability-indicating RP-HPLC method for simultaneous estimation of PER, IND, and AML in bulk and fixed-dose combination. It is pertinent to note that all the published methods enabled estimation of a single drug or combination of either PER and IND or PER and AML dosage forms only. One HPLC method is reported to separate PER, IND, and AML but does not include any work on stress testing [39]. Hence, RP-HPLC chromatographic conditions were developed by applying forced degradation studies, and the method was validated to establish selectivity with respect to potential degradation products.

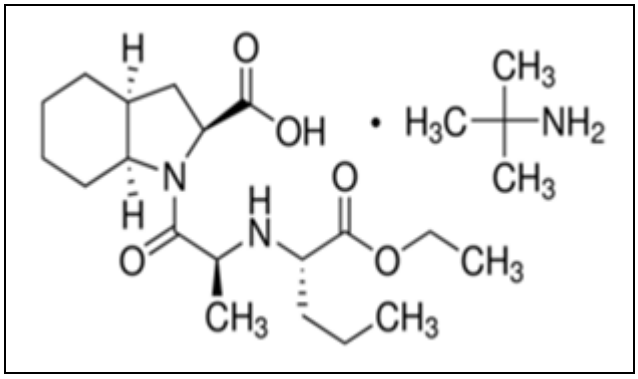

FIG. 1: STRUCTURE OF PERINDOPRIL ERBUMINE

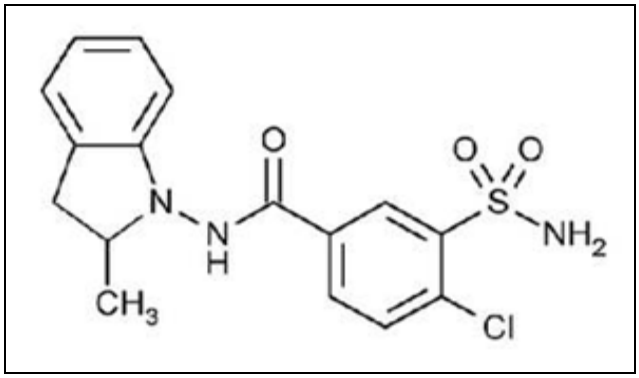

FIG. 2: STRUCTURE OF INDAPAMIDE

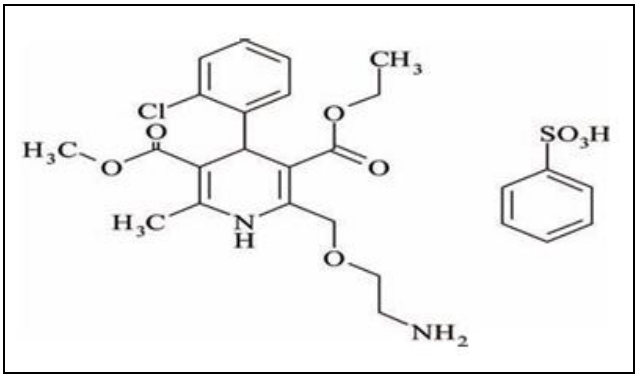

FIG. 3: STRUCTURE OF AMLODIPINE BESYLATE

MATERIALS AND METHODS:

Apparatus: Chromatographic separation of drugs was performed on a Shimadzu HPLC instrument (LC_2010 CHT) [software LC Solution, equipped with a photodiode array detector (SPD-M20A), Auto-sampler]. The system contains a quaternary gradient pump, autosampler, column oven, and a PDA detector.

The software referred to above was used to record and integrates the chromatograms. Among other instruments, analytical balance (Acculab ALC-210.4, Huntingdon Valley, PA), Photostability chamber (TH-90S, Thermolab, Mumbai, India), Hot air oven (TO-90S, Thermolab), pH meter (Thermo Electron Crop., Pune, India), Sonicator (EN 30 US, Enertech Fast Clean, Mumbai, India) were also used at different stages of development and validation of stability-indicating assay method.

Chemicals and Reagents: The API of Perindopril tributylamine, Indapamide, and Amlodipine Besylate were provided as gift samples from Emcure Pharmaceuticals LTD, IPCA Laboratories LTD, and West Coast Pharmaceuticals, respectively. Tablets TRIPLIXAM 10/2.5/10 (manufactured by Servier) were imported from the international market. All the solvents like Methanol, Acetonitrile, and Sodium dihydrogen orthophosphate buffer were of HPLC grade from Finar Chemicals Ltd, Ahmedabad, India. Water was also of HPLC grade from RFCL Limited, New Delhi, India. AR grade orthophosphoric acid (OPA) and Decanesulphonic acid were from SD Fine Chemicals Pvt. Ltd., Ahmedabad, India. All the chemical reagents were of analytical grade.

Preparation of Standard Stock Solution: The standard solutions of PER and AML were prepared by weighing accurately 20.344 mg PER erbumine equivalent to 25 mg PER arginine and 34.67 mg Amlodipine besylate equivalent to 25 mg AML individually and transferred into a clean and dry 25 ml volumetric flask.

The volume was made up to the mark with the methanol to achieve 1000 µg/mL standard stock solution, respectively. The standard solutions of IND was prepared by weighing accurately 25 mg IND and transferred into clean and dry 100 ml volumetric flask. The volume was made up to the mark with the methanol to achieve 250 µg/mL standard stock solution of IND.

Preparation of Test Solution: Twenty TRIPLIXAM tablets were accurately weighed, their average weight was calculated. Amount of finely powdered tablet equivalent to 10 mg PER Arginine, 2.5 mg IND, and 10 mg AML were weighed and transferred into a 10 mL volumetric flask, and the volume was adjusted to mark with methanol. The content of the flask was sonicated for 30 min to dissolve the active ingredients completely. The solution was filtered through a Whatman filter paper no. 41. From this, 2 mL aliquot was transferred into a 20 mL volumetric flask and the volume was made with diluent. This test sample solution containing working concentrations of 100 µg/mL PER Arginine, 25 µg / mLIND & 100 µg/m LAML, respectively, was analyzed with the optimized chromatographic condition for assay determination.

Preparation of Mobile Phase: About 0.78 g of sodium dihydrogen phosphate and 300 mg of decanesulphonic acid salt were dissolved into 1000 ml of water. The pH of this solution was adjusted to 2.0 with orthophosphoric acid. For the mobile phase, a mixture of Buffer (A) and ACN (B) in gradient ratio of (0 min 85:15 %V/V, 50 min 50:50 % V/V, 55 min 85:15 % V/V, 65 min 85:15% V/V) were used.

Standardized Chromatographic conditions: The analyte drugs and degradation products were well separated with ZORBAX RX C8 (250 mm × 4.6 mm, 5 µm) as a stationary phase and gradient system of Sodium dihydrogen phosphate buffer of pH 2 with OPA and decanesulphonic acid (A) and Acetonitrile (B). The flow rate was adjusted to 1 mL/min. The determination was done at wavelength 215 nm as PER does not have significant absorbance above 220 nm. Column temperature was set to 40 ºC. The total run time was 70 min. The injection volume was 10 µL. A combination of water, Acetonitrile, and Orthophosphoric acid in ratio of 500:500:1 was used as a diluent.

Method Validation: The proposed method was validated as per ICH guidelines Q2 R1.

System Suitability Test Parameters: System suitability tests are used to verify that the resolution and repeatability of the system were adequate for the analysis intended.

The parameters used in this test were the chromatographic peak resolution (>2), theoretical plate number (>2000), and tailing factor (<1.8). The repeatability of these parameters was checked by injecting six solutions of PER, IND, and AML.

Linearity: From the standard stock solution containing 1000 µg/mL PER, 250 µg/mL IND and 1000 µg/mL AML, aliquots of 1 mL, 1.6 mL, 2 mL, 2.2 mL, 2.4 mL and 3 mL were transferred in clean and dry 20 mL volumetric flasks respectively. The volume was made up to the mark with diluent. This yielded solution of 50, 80, 100, 110, 120 and 150 µg/mL of PER and AML and 12.5, 20, 25, 27.5, 30 and 37.5 µg/mL of IND respectively. An injection volume of 10 µL of each solution was injected under operating chromatographic conditions. Six replicates of each concentration were performed, and then calibration plots were determined by linear least – squares regression. The plate was developed on the previously described mobile phase. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs.

Precision: Repeatability was determined by applying six replicates of test solution (100 µg/mL PER, 25 µg/mL IND, and 100 µg/mL AML). The intraday and inter-day precisions were determined by responses of six replicates on the same and different days for the test concentration. The results were reported in terms of % RSD.

Accuracy: Recovery study was carried out by standard addition method where a known amount of standard concentration at 50%, 100%, and 150% of the test solution were spiked in the test solution in triplicate. The amount of drugs was estimated by substituting values in the regression equation. The % RSD of the recovery was calculated.

LOD and LOQ: The LOD and LOQ of the developed method were calculated from the calibration curve using equations,

$$LOD = 3.3 \times \sigma/S \text{ and } LOQ = 10 \times \sigma/S$$

Where σ is the standard deviation of y-intercept and S is the slope of the curve.

Robustness: By introducing small changes in the Flow rate (± 0.1 mL), column temperature (± 5 ºC), wavelength (± 2 nm), and pH of buffer solution in the mobile phase (± 0.2); the effects on the results were determined. One factor at a time was changed, and the effect on peak area of the drug was studied. The robustness of the method was done on a single level for six replicates, and % RSD was calculated.

Specificity: The specificity of the method was checked by the peak purity of analyte peaks and also the adequate resolution of analyte peaks in forced degradation samples.

Forced Degradation Studies: Force degradation study was intended to ensure the effective separation of PER, IND, and AML and their degradation products which are generated under different stress conditions like acid, alkaline and neutral hydrolysis, oxidative degradation, thermal and photolytic degradation.

Acid Hydrolysis: Accurately weighed 10 mg PER, 2.5 mg IND, and 10 mg AML were transferred in 10 mL volumetric flask individually and in combination. To this were added 5 mL methanol and 5 mL of 0.1 N HCl, and it was kept at 70 ºC for 3 h. From that solution, 1 mL was transferred into 10 mL volumetric flask, neutralized with 0.2 N NaOH and diluted to mark with diluent. This corresponds to100 µg/ mL PER, 25 µg/ mL IND and 100 µg/ mL AML were injected under operating condition.

Alkaline Hydrolysis: Accurately weighed 10 mg PER, 2.5 mg IND and 10 mg AML were transferred in 10 mL volumetric flask individually and in combination. To this were added 5 mL methanol and 5 mL of 0.1 N NaOH and it was kept at 70 ºC for 3 h.

From that solution, 1 mL was transferred into 10 mL volumetric flask, neutralized with 0.1 N HCl, and diluted to mark with diluent. This corresponds to100 µg/ mL PER, 25 µg/ mL IND and 100 µg/ mL AML were injected under operating condition.

Neutral Hydrolysis: Accurately weighed 10 mg PER, 2.5 mg IND, and 10 mg AML were transferred in 10 mL volumetric flask individually and in combination. To this were added 5 mL methanol and 5 mL of water, and it was kept at 70 ºC for 3 h. From that solution, 1 mL was trans-ferred into 10 mL volumetric flask and diluted to mark with diluent. This corresponds to100 µg/ mL PER, 25 µg/ mL IND and 100 µg/ mL AML were injected under operating condition.

Oxidative Hydrolysis: Accurately weighed 10 mg PER, 2.5 mg IND, and 10 mg AML were transferred in 10 mL volumetric flask individually and in combination. To this were added 5 mL methanol

and 5 mL of 3% H_2O_2, and it was kept at RT for 12 h. From that solution, 1 mL was transferred into 10 mL volumetric flask and diluted to mark with diluent. This corresponds to100 µg/ mL PER, 25 µg/ mL IND and 100 µg/ mL AML were injected under operating condition.

Thermal Degradation: Accurately weighed quantity of 10 mg PER, 2.5 mg IND, and 10 mg AMLO were kept individually and in combination in petridish. Those were kept at 700 C for 6 h. After that, those were dissolved in 10 mL methanol. From that solution, 1 mL was transferred into 10 mL volumetric flask and diluted to mark with diluent. This corresponds to100 µg/ mL PER, 25 µg/ mL IND and 100 µg/ mL AML were injected under operating condition. The same condition was applied to the formulation, and solutions were prepared with the above-mentioned concentrations according to the dilution scheme.

Photolytic Degradation: Accurately weighed quantity of 10 mg PER, 2.5 mg IND, and 10 mg AML were kept individually and in combination in petridish. It was exposed in a photostability chamber (TH-90S, Thermo lab, Mumbai, India) in UV light at 254 nm for 12 h to get 200 watt-hours/m^2 intensity. It was dissolved in 10 mL methanol. From that solution, 1 mL was transferred into 10 mL volumetric flask and diluted to mark with diluent. This corresponds to100 µg/ mL PER, 25 µg/ mL IND and 100 µg/ mL AML were injected under operating condition. The same condition was applied to the formulation, and solutions were prepared with the above-mentioned concentrations according to the dilution scheme.

RESULTS AND DISCUSSION:

Optimized Chromatographic Condition: For separation of PER, IND and AML, and their degradation peaks, different mobile phases with different solvents in different ratio were tried like (1) Methanol : Buffer (pH 6.0) (55:45 %V/V) (2) Acetonitrile: Methanol: Buffer (pH 4.5) (20:20:60 % V/V/V) (3) Acetonitrile: Buffer (pH 3.5) (70:30 %V/V) (4) Buffer (pH 2.5) with TEA : Acetonitrile in a gradient run (0 min 90:10%V/V, 15 min 65:35 % V/V, 55 min 50:50% V/V, 60 min 90:10% V/V, 70 min 90:10% V/V).

During the trials, it was observed that the mobile phase in gradient form was required to bring about a separation. The addition of decanesulfonate, an anionic ion-pairing agent, further improved the retention and resolution of three analyte drugs. Decane-sulphonate formed in pairs with protonated drugs and improved retention. The gradient proportion was fixed as follows: a mixture of Buffer (pH 2) with Decanesulphonic acid (A): Acetonitrile (B) in gradient ratio of (0 min 85:15% V/V, 50 min 50:50% V/V, 55 min 85:15% V/V, 65 min 85:15% V/V) showed a well-resolved peak with better peak shape.

The drugs were resolved with t_R of 31.39 ± 0.21 min, 25.73 ± 0.41 min, and 36.95 ± 0.47 min of PER, IND, and AML, respectively. Determination of all three drugs was done at wavelength 215 nm with column temperature 40 ºC and adjusted flow rate 1.0 mL/min. Injection Volume was set 10 µL for a good peak shape **Fig. 4**.

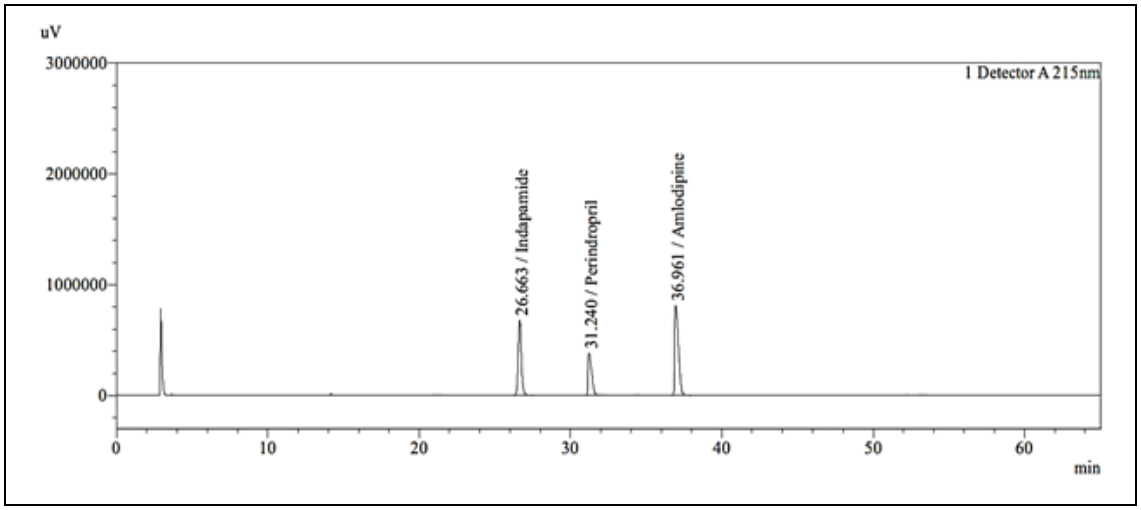

FIG. 4: CHROMATOGRAM OF STD PER (50 µg/ml), IND (12.5 µg/ml) AND AML (50 µg/ml)

System Suitability Test Parameters: The result of the system suitability test, like the number of theoretical plates, tailing factor, and resolution, were found within the acceptable range, which indicates that the system was suitable for the intended analysis **Table 1**.

TABLE 1: SYSTEM SUITABILITY PARAMETERS FOR PER, IND AND AMLO

Parameter	PER	IND	AML
Retention time	31.39 ± 0.21 min	25.73 ± 0.41 min	36.95 ± 0.47 min
Theoretical Plates	115919.16 ± 1817.97	64020.17 ± 327.82	166034 ± 605.49
Tailing Factor	1.495 ± 0.01	1.125 ± 0.01	1.555 ± 0.01
Resolution	15.62 ± 0.21	--	15.41 ± 0.185

Linearity: The method was found linear over the concentration range of 50-150 µg/ mL, 12.5-37.5 µg/ mL and 50-150 µg/ mL for PER, IND and AML respectively.

The calibration curve obtained by the least square regression analysis between average peak area and concentration showed a linear relationship with a correlation coefficient of 0.9995, 0.9998, and 0.9992 for PER, IND, and AML, respectively.

The linear regression equation were y = 72909x + 281723, y = 450475x + 262256 and y = 161775x + 729106 for PER, IND and AML respectively **Table 2**, **Fig. 5**, **6** and **7**.

TABLE 2: LINEARITY RANGE OF PER, IND AND AMLO

S. no.	PER			IND			AML		
	Conc (µg/ mL)	Peak Area*±SD	%RSD	Conc (µg/ mL)	Peak Area*±SD	% RSD	Conc (µg/ mL)	Peak Area*±SD	% RSD
1	50	3864206 ± 5539.495	0.88	12.5	5914217 ± 7888.87	0.63	50	8607930 ± 4233.394	0.95
2	80	6219247 ± 16121.98	0.79	20	9193948 ± 30047.51	0.75	80	13836849 ± 21556.7	1.54
3	100	7579129 ± 20066.51	0.64	25	11515424 ± 29713.24	0.96	100	17021149 ± 43734.06	0.76
4	110	8262338 ± 22311.6	0.91	27.5	12722540 ± 34818.81	1.27	110	18561978 ± 50871.83	1.17
5	120	9037664 ± 34966.83	1.25	30	13806123 ± 48990.26	1.35	120	20213800 ± 143161.2	1.20
6	150	11202134 ± 37736.76	1.32	37.5	17118665 ± 58426.33	1.17	150	24815480 ± 278100.5	1.12

*Average of six determinations

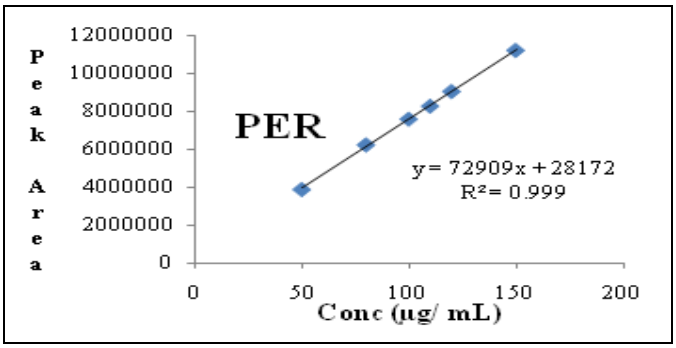

FIG. 5: CALIBRATION CURVE OF LINEARITY OF PER

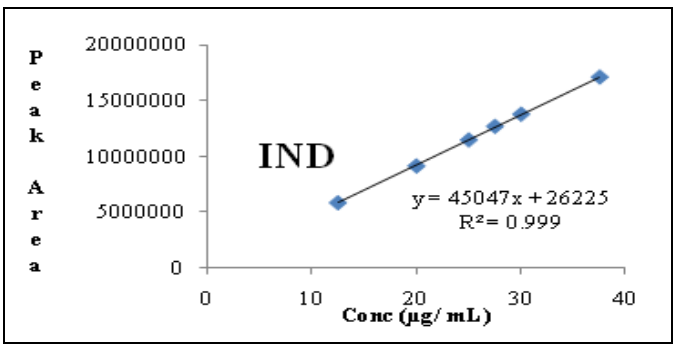

FIG. 6: CALIBRATION CURVE OF LINEARITY OF IND

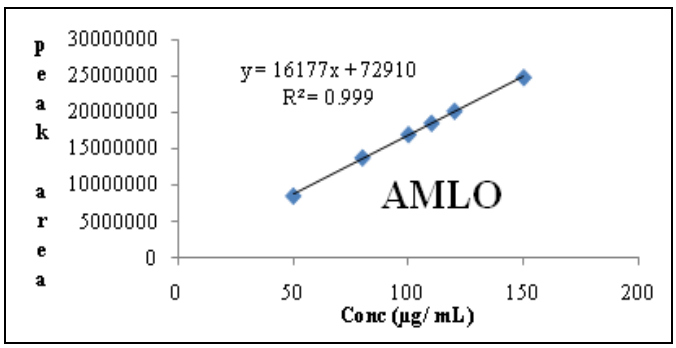

FIG. 7: CALIBRATION CURVE OF LINEARITY OF AML

Precision: The repeatability was found to be satisfactory with % RSD of 0.87for PER, 1.63 for IND, and 1.06 for AML. The Intraday Precision was found to be satisfactory with % RSD of 1.17 for PER, 1.32 for IND, and 1.22 for AML. The Interday precision was found to be satisfactory with % RSD of 1.45 for PER, 0.83 for IND, and 1.09for AML. Hence, confirming the precision of the developed method **Table 3**.

TABLE 3: REPEATABILITY, INTRADAY AND INTERDAY PRECISION OF PER, IND AND AMLO

Parameter	PER			IND			AML		
	Conc (µg/ mL)	Peak Area* ± SD	% RSD	Conc (µg/ mL)	Peak Area* ± SD	% RSD	Conc (µg/ mL)	Peak Area* ± SD	% RSD
Repeatability	100	7551450.67 ± 65962.38	0.87	25	11595299.5 ± 189695.25	1.63	100	16993927 ± 180671.13	1.06
Intraday Precision	100	7546640.67 ± 88089.41	1.17	25	11622314.83 ± 153196.38	1.32	100	17009876.67 ± 208489.28	1.22
Interday Precision	100	7564729.33 ± 109720.72	1.45	25	11661281.50 ± 97544.25	0.83	100	17002908.83 ± 185152.54	1.09

*Average of six determinations

Accuracy: The accuracy of the developed method was established by the standard addition method by adding known standard concentration solutions to the pre-analyzed samples. Recoveries were in between 99.34 – 101.06% for PER, 99.46 - 100.00% for IND and 98.61 – 101.01 % for AML, which is according to guidelines that prove method to be accurate **Table 4**.

TABLE 4: RECOVERY STUDY DATA OF PER, IND AND AMLO

Drug	Amount of Test Solution (µg/mL)	Amount of Std added (µg/mL)	Peak area* ± SD	Amount Found (µg/mL)	Recovery (µg/mL)	% Recovery	% RSD
PER	50	0	3944985 ± 35659.65	50.24	0	100.49	0.97
	50	25	5769277 ± 22696.02	75.27	25.27	101.06	1.23
	50	50	7556009 ± 29100.72	99.77	49.77	99.54	0.80
	50	75	9359262.67 ± 84653.79	124.51	74.51	99.34	1.56
IND	12.5	0	5862696.67 ± 34960.32	12.43	0	99.46	0.62
	12.5	6.25	8708737.33 ± 32262.38	18.75	6.25	100	1.15
	12.5	12.5	11497919.33 ± 34217.10	24.94	12.44	99.53	0.61
	12.5	18.75	14300624.67 ± 48190.33	31.16	18.66	99.54	0.57
AML	50	0	8754517.33 ± 85202.24	49.61	0	99.22	1.06
	50	25	12806177.33 ± 47364.72	74.65	24.65	98.61	1.19
	50	50	16923355 ± 143777.87	100.1	50.1	100.21	1.77
	50	75	21074263.33 ± 74143.14	125.76	75.76	101.01	0.60

*Average of three determinations

TABLE 5: ROBUSTNESS STUDY OF PER, IND AND AML

Condition	Variation	%Assay* ± SD			% RSD#		
		PER	IND	AML	PER	IND	AML
Normal	NA	99.98 ± 1.11	100.30 ± 1.75	99.79 ± 1.05	1.22	1.28	1.11
Flow rate	1.1 mL/min	100.32 ± 1.11	101.2 ± 1.74	99.98 ± 1.05			
(± 0.1 mL/min)	0.9 mL/min	100.02 ± 0.83	101.67 ± 1.03	99.52 ± 0.69			
Column temperature	55 ºC	99.43 ± 1.58	100.58 ± 1.05	98.94 ± 1.14			
(± 5 ºC)	45 ºC	100.61 ± 1.01	101.1 ± 1.22	98.54 ± 0.79			
Wavelength	217 nm	101.23 ± 1.23	99.45 ± 1.34	98.12 ± 1.29			
(± 2 nm)	213 nm	101.86 ± 1.45	99.75 ± 1.26	98.45 ± 1.31			
pH (± 0.2)	4.1	100.85 ± 1.19	101.15 ± 1.18	98.74 ± 1.49			
	3.7	101.15 ± 1.36	101.69 ± 1.43	101.14 ± 1.12			

*Average of six determinations #RSD of original and modified conditions

LOD and LOQ: The LOD calculated by formulae was found to be 0.99 µg/ mL for PER, 0.87 µg/ mL for IND, and 3.98 µg/ mL for AML. The LOQ calculated by formulae was found to be 3.02 µg/ mL for PER, 2.6 µg/ mL for IND and 12.08 µg/ mL for AML.

Robustness: Slight change in the chromatographic condition of the developed method like small changes in the Flow rate (± 0.1 mL), column temperature (± 5 ºC), wavelength (± 2 nm), and pH of buffer solution in the mobile phase (± 0.2) did not affect the result significantly.

The % RSD values were found below 2 indicated the method to be robust **Table 5**.

Analysis of Marketed Formulation: The developed method was applied to marketed tablet preparation. The assay results of PER arginine, IND, and AML were 99.98 ± 1.11%, 100.30 ± 1.75%, and 99.79 ± 1.05%, respectively, of the labeled amount **Table 6** and **Fig. 8**.

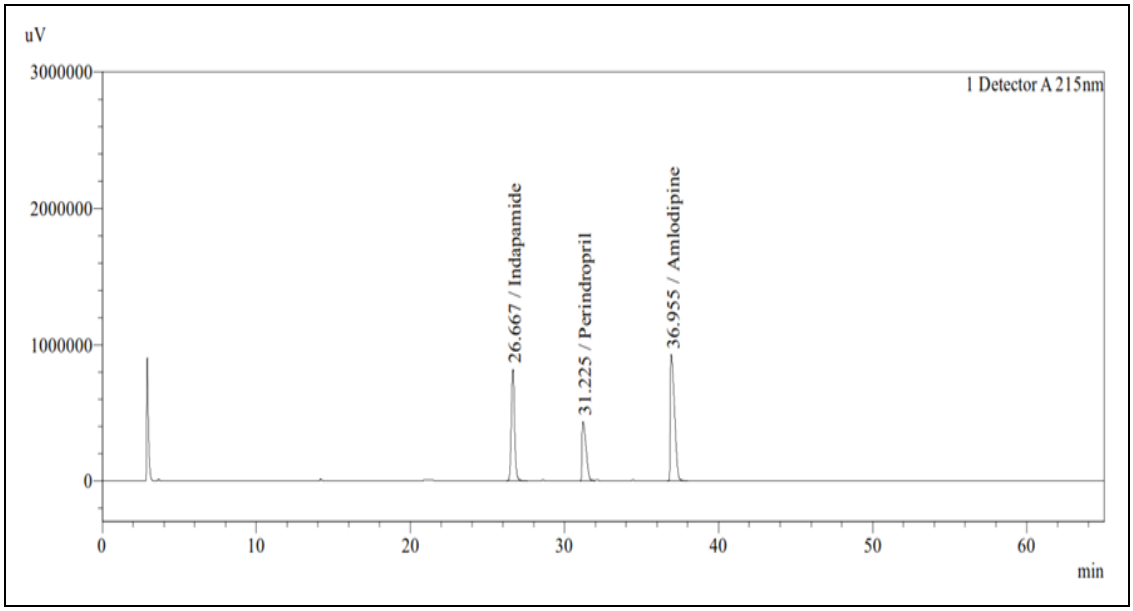

FIG. 8: CHROMATOGRAM OF TEST SOLUTION CONTAINING PER (50 µg/ ml), IND (12.5 µg/ ml) AND AML (50 µg/ ml)

TABLE 6: ANALYSIS OF MARKETED FORMULATION

Drug	Amount of Drug (mg)		% Label claimed* ± SD	% RSD
	Labeled	Estimated		
PER	10	9.99	99.98 ± 1.11	1.11
IND	2.5	2.51	100.30 ± 1.75	1.74
AML	10	9.98	99.79 ± 1.05	1.05

*Average of six determinations

Specificity: The method was found to be specific with respect to excipients in a test sample as no interfering peaks were found. The specificity with respect to potential degradation products was established by performing force degradation and analyzing the samples. The specificity was further confirmed by peak purity (match factor) of 1000, 1000, and 1000 for PER, IND, and AML, respectively, for analyte peaks in the test sample and stress samples.

Forced Degradation Studies: The result of forced degradation studies are summarized in the table. Under the optimized chromatographic conditions, the analyte drug peaks were well resolved from potential degradation products, and the percent degradation was calculated by comparing peak area with standard preparation. During forced degradation studies, PER degraded significantly under acidic, alkaline, oxidative, and thermal stress conditions; degraded moderately under photolytic stress conditions; and degraded the least under neutral stress conditions. All four degradation products were eluted in different stress conditions. Two referred article revealed the formation of only Impurity F in thermal condition. Our study also found one degradation product under thermal condition corresponding to t_R 38.707 min, which could be attributed to Impurity F. The same article reported the formation of perindoprilat (Impurity B refers to EP) in acidic, alkaline, oxidative, and thermal stress conditions but majorly in acidic hydrolysis. Our study also found one degradation product under acidic, alkaline, and oxidative stress conditions corresponding to t_R 19.33 min; hence it can be attributed to Impurity B (Perindoprilat). The article reported the formation of Impurity D majorly under alkaline hydrolysis. Our study found two degradation products under alkaline hydrolysis corresponding to t_R 13.17 min and 21.64 min, one of these may be attributed to Impurity D.

IND degraded significantly under acidic, alkaline and oxidative stress conditions, degraded moderately under neutral stress conditions and degraded the least under thermal and photolytic stress conditions. In all 3 degradation products were resolved in different conditions. One published paper indicated the formation of only Impurity B refers to EP in thermal stress conditions.

Our study also found only one degradation product corresponding to t_R 30.497 min under thermal stress conditions. This can therefore be attributed to Impurity B. The above-referred paper and other referred articles revealed formation of 4-chloro-3-sulfamoyl benzoic acid (CSBA), and Impurity B refers to EP in acidic, alkaline, and oxidative stress conditions. Our study also found two degradation products corresponding to t_R 4.677 min and 30.453 min, commonly under acid, alkaline, and oxidative stress conditions; hence those can be attributed to being CSBA and Impurity B, respectively.

AML degraded significantly under acidic, alkaline, and oxidative stress conditions degraded moderately under photolytic stress conditions and showed negligible degradation under neutral stress conditions. In all total nine degradation products were found in different stress conditions. Two reliable published papers indicated the formation of dihydro derivative (Impurity D as per EP) under acidic hydrolysis and oxidative stress conditions. Our study also found 1 degradation product corresponding to t_R 31.20 min under acidic hydrolysis and oxidative stress conditions. This can therefore be attributed to dihydro derivative.

Article 3 revealed some common degradation products under acidic and alkaline hydrolysis like AM1, AM6, and AM9. Our study also found 1 degradation product common for acid and alkali hydrolysis corresponding to t_R 34.48 min, which can be attributed to one of the above. One of the above referred article reported the formation of 1 degradation product due to acetyl group under alkaline hydrolysis. Our study found two degradation pro-ducts corresponding to t_R 14.25 min and 16.267 min, one of which could be due to the acetyl group.

The method is also deemed to be specific with respect to potential degradation products as all the observed degradation products were adequately resolved from all three analyte peaks. The impurities were found under acidic degradation, alkaline hydrolysis, oxidative degradation, and thermal stress conditions. The sample tablet was exposed to thermal and photolytic degradation conditions as per ICH Q1A R2 guidelines, and the results were comparable to the standard mixture. The method was therefore considered to be Stability indicating for tablet solid dosage form **Table 7**, **Table 8,** and **Fig. 9-14**.

TABLE 7: SUMMARY OF FORCED DEGRADATION STUDY OF INDIVIDUAL API IN MIXTURE OF PER, IND AND AML

Degradation condition	t_R of Analyte			Match factor*			No. of degradation peaks	t_R of Degradation peak	% Degradation		
	PER	IND	AML	PER	IND	AML			PER	IND	AML
0.1 N HCl at 70 ºC, 3 h	31.657	25.560	37.373	993	1000	1000	8	7.073, 19.347, 29.193, 30.453, 33.827, 34.60, 38.693, 47.627	20.53	15.23	20.83
0.1 N NaOH at 70 ºC, 3 h	31.697	25.543	37.340	998	1000	1000	8	4.677, 13.14, 14.21, 16.22, 19.157, 21.040, 30.497, 34.48	27.32	18.70	21.52
Water, 70 ºC, 3 h	31.707	25.61	37.380	1000	1000	1000	--	--	4.67	12.04	2.98
3% H_2O_2, RT, 12 h	31.657	25.507	37.307	1000	1000	1000	5	4.647, 6.95, 13.503, 20.883, 30.507	23.52	22.25	18.07
Thermal at 70 ºC, 6 h	31.673	25.567	37.370	1000	1000	1000	4	13.063, 21.610, 30.497, 38.707	31.57	7.24	8.04
UV light, 254 nm, 24 h	31.673	25.570	37.370	1000	1000	1000	--	--	9.93	5.93	8.67

*Match factor between 990 to 1000 shows identical peaks

TABLE 8: SUMMARY OF FORCED DEGRADATION STUDY IN PHARMACEUTICAL DOSAGE FORM

Degradation condition	t_R Value of Analyte			Match factor*			No. of degradation peaks	t_R of Degradation Peak	% Degradation		
	PER	IND	AML	PER	IND	AML			PER	IND	AML
Thermal at 70 ºC, 6 h	31.707	25.59	37.377	1000	1000	1000	4	13.090, 21.610, 30.514, 38.546	29.33	6.91	9.77
UV light, 254 nm, 24 h	31.673	25.66	37.373	1000	1000	1000	--	--	8.45	6.87	5.46

*Match factor between 990 to 1000 shows identical peaks

TABLE 9: SUMMARY OF VALIDATION PARAMETERS

S. no.	Parameter	PER	IND	AML
1	Specificity	Specific	Specific	Specific
2	t_R value	31.39 ± 0.21 min	25.73 ± 0.41 min	36.95 ± 0.47 min
3	Linearity Change	50-150 µg/ mL	12.5-37.5 µg/ mL	50-150 µg/ mL
4	Regression Line equation	y = 72909x + 281723	y = 450475x + 262256	y = 161775x + 729106
5	Correlation Coefficient	0.9995	0.9998	0.99920
6	Precision (% RSD) Repeatability	0.87	1.63	1.06
	Intraday Precision	1.17	1.32	1.22
	Interday Precision	1.45	0.83	1.09
7	Accuracy (% Assay)	99.34 – 101.06	99.46 - 100.00	98.61 – 101.01
8	LOD (µg/ mL)	0.99 µg/ mL	0.87 µg/ mL	4.00 µg/ mL
9	LOQ (µg/ mL)	3.02 µg/ mL	2.6 µg/ mL	12.08 µg/ mL
10	Robustness	Robust	Robust	Robust

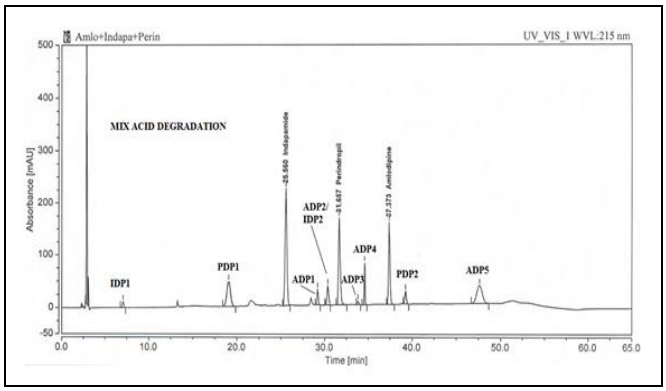

FIG. 9: CHROMATOGRAM OF ACID HYDROLYSIS IN MIXTURE OF PER, IND AND AML

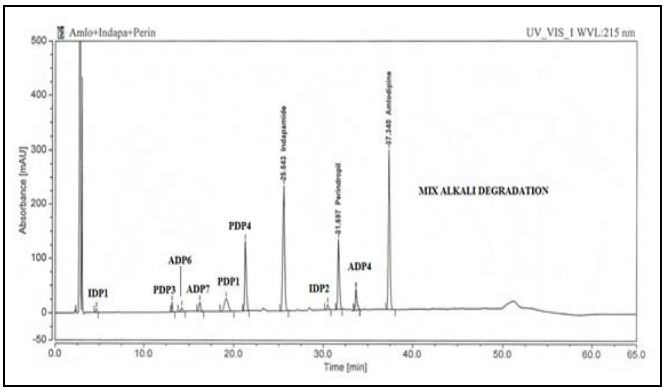

FIG. 10: CHROMATOGRAM OF ALKALINE HYDROLYSIS IN MIXTURE OF PER, IND AND AML

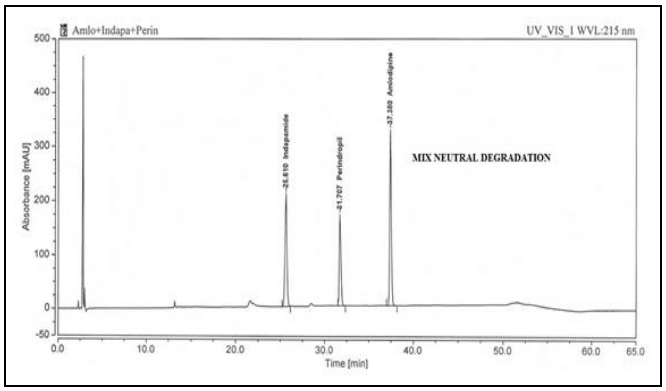

FIG. 11: CHROMATOGRAM OF NEUTRAL HYDROLYSIS IN MIXTURE OF PER, IND AND AML

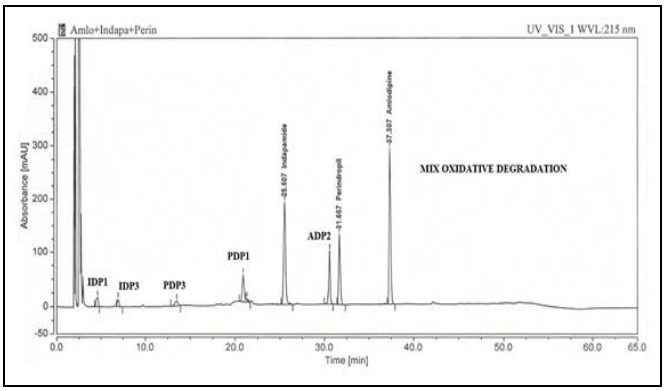

FIG. 12: CHROMATOGRAM OF OXIDATIVE DEGRADATION IN MIXTURE OF PER, IND AND AML

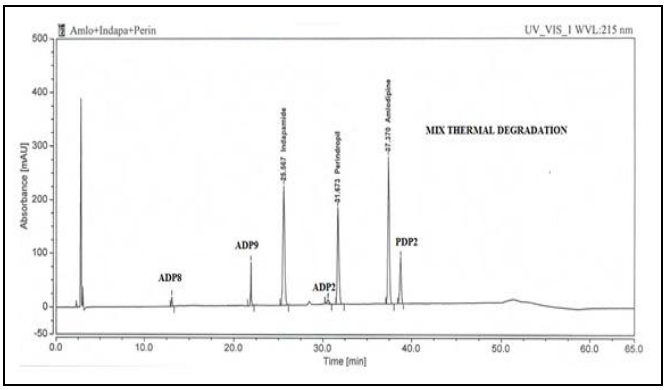

FIG. 13: CHROMATOGRAM OF THERMAL DEGRADATION IN MIXTURE OF PER, IND AND AML

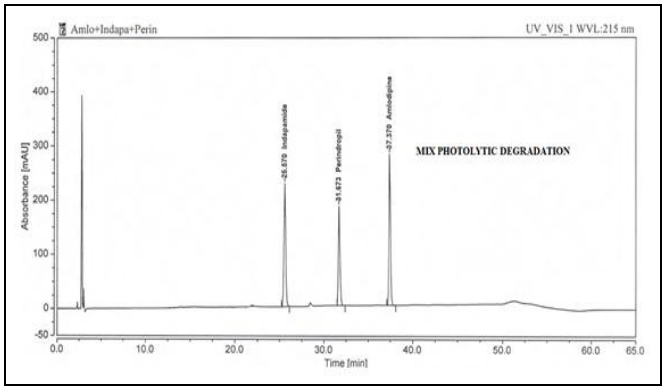

FIG. 14: CHROMATOGRAM OF PHOTOLYTIC DEGRADATION IN MIXTURE OF PER, IND AND AML

CONCLUSION: The proposed RP-HPLC method is precise, specific, linear, and accurate for the estimation of PER, IND, and AML in any pharmaceutical dosage form without interference from the excipients and potential degradation products formed in various stress conditions like acid hydrolysis, alkaline hydrolysis, neutral hydrolysis, oxidative, thermal and photolytic degradation conditions.

The results of stress testing were critically analyzed to establish a correlation with degradation products reported in published literature. The developed method is validated as per ICH guidelines. The results showed the suitability of the developed method for degradation kinetic studies and stability studies of the fixed dose combination.

A method can also be suitably applied for the estimation of FDC containing two drugs like PER and IND and PER with AML. The use of the method can be extended for estimation of one or more degradation products as all the degradation products were found adequately separated from one another and also from drug peaks.

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