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Stability-Indicating Rp-Uplc Method for the Simultaneous Estimation of Perindopril Arginine and Amlodipine Besylate in Pharmaceutical Formulation in the Presence of their Degradation Products

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Abstract

A new stability-indicating chromatographic ultra-performance liquid chromatography (UPLC) method has been developed and validated for determination of Perindopril Arginine (PER) and Amlodipine Besylate (AML) in their dosage form and in the presence of their degradation products. The method was based on ultra-performance liquid chromatography, by which the components were separated on a Acquity UPLC BEH C18, 1.7 µm, 2.1 × 50 mm. part no.186002350 column, using a mobile phase consisting of phosphate buffer pH 2 and acetonitrile (75:25) with ultraviolet detection at 210 nm. Different parameters that affect this new method were optimized to achieve maximum separation for those components. Both drugs were subjected to forced degradation studies under hydrolysis (acidic, alkaline and oxidative stress). The proposed method is considered as stability indicating because of the resolution of the aforementioned drugs from their degradation products. Moreover, the degradation products for both drugs were subjected to LC-MS for structure elucidation. System suitability parameters of the developed method were also tested.

Keywords: UPLC; Perindopril arginine; Amlodipine besylate; Stress testing; Structure elucidation

Introduction

Perindopril Arginine (PER), (2S,3aS,7aS)-1-[(2S)-2-[[(1S)-1-(ethoxycarbonyl) butyl] amino]-1-oxopropyl] octahydro-1H-indole-2-carboxylic acid, is an angiotensin converting enzyme inhibitor (ACE inhibitor) that is used in the treatment of heart failure and hypertension. It can be used in case of patients with stable ischaemic heart disease to reduce the risk of cardiovascular events in [1]. PER is converted into its active metabolite perindoprilate in the body. Amlodipine Besylate (AML), 2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylic acid 3-ethyl 5-methyl ester, has calcium antagonist activity as it is considered as one of the dihydropyridine derivative group [1]. It is used in the treatment of prinzmetal variant angina, chronic stable angina pectoris and hypertension [2]. Nowadays, PER has been

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marketed in combination with AML in tablets for the treatment of essential hypertension. This combination is advised for patients whose blood pressure is not adequately controlled by either drug alone, as it has been shown to be superior in lowering diastolic and systolic blood pressures when compared with either of the monotherapy regimens. This combination also has significantly fewer dose-dependent adverse experiences than high-dose calcium antagonist monotherapy [3]. PER is formulated either as perindopril erbumine or as perindopril arginine. The advantage of arginine salt over erbumin is that it imparts certain stability to perindopril and inhibits the cyclyzation process in open containers or at high temperatures [4].

A literature survey revealed that both amlodipine besylate and perindopril erbumine salt are official in the British Pharmacopoeia [5]. However, perindopril arginine and its pharmaceutical combination with amlodipine besylate (Amloveran tablet) is not officially in any pharmacopeia. A detailed literature survey revealed that perindopril erbumine has been determined in biological fluids and pharmaceutical formulations either in binary mixtures, enantiomeric mixtures or in the presence of its metabolite with different techniques such as thin-layer chromatography (TLC) [6-8], high-performance liquid chromatography (HPLC) [9,10], LC [11,12], gas chromatography (GC) [13-15], potentiometric selective electrodes [16-18], capillary zone electrophoresis (CZE) [19-21], selective biosensors [22-24], spectrophotometry [25,26] and radio-immunoassay (RIA) [27]. Amlodipine besylate has been assayed using HPLC [28-30], TLC [31-33], capillary electrophoresis (CE) [34-36], LC mass spectrometry (MS)-nuclear magnetic resonance (NMR), electrospray ionization (ESI) [37,38] and voltammetric techniques [39,40] both in binary and enantiometric mixtures. Also there were two methods for perindopril erbumine and amlodipine besylate by spectrophotometric techniques using absorption factor method and the other one used HPLC technique [41,42]. Also there was HPLC method and densitometry method for simultaneous determination of Perindopril Arginine and amlodipine Besylate in solid dosage form [43,44]. There is no stability-indicating assay UPLC method for the determination of both PER and AML in their dosage form. Accordingly, the objective of this manuscript is to establish a validated UPLC stability-indicating method for the determination of both active compounds in the presence of their degradation products.

Materials and Methods

Materials

PER was supplied by Glenmark, batch number 80150956 with potency of 99.24. AML was supplied by Lee Pharma, batch number ABFP15001 with a potency of 99.17. Amloveran 10/10 mg tablets (EVA Pharma for pharmaceuticals and medical appliances), each tablet labeled to contain 13.85 mg of Amlodipine Besylate (equivalent to 10 mg Amlodipine) and 10 mg Perindopril Arginine. They were purchased from local market. Acetonitrile, potassium dihydrogen phosphate, and O-phosphoric acid 85%, triethylamine, methanol and hydrochloric acid were obtained from MERCK, Also, 1.0 N NaOH, 1.0 N HCl and 3% H_2O_2 were prepared.

Instrument

The liquid chromatography system consisted of Waters Acquity H UPLC class with sample manger-FTN, Quaternary solvent manager, and column oven and photodiode array (PDA) detector. UPLC MS/MS Agilent system consisted of triple quad 6420 detectors, quaternary pump 1290 infinity, infinity auto sampler 1290 and mass hunter software.

Chromatographic conditions

The column that used for UPLC method was 50 × 2.1 mm i.d. acquity UPLC BEH C18 analytical column and it was obtained from Waters, while the column used for LC-MS was Phenomenex, Kinetex 2.6 µm C18, 50 × 4.6 mm. The mobile phase consisted of phosphate buffer (prepared by adding 6.8 g of potassium dihydrogen phosphate anhydrous in 900 ml water then add 3 ml triethylamine and adjust pH to 2 with phosphoric acid and complete to 1000 ml) and acetonitrile with the ratio of (75:25). The mobile phase was filtered through a 0.2 µm Pall membrane then degassed for 15 min in an ultrasonic bath before use. The samples were filtered also through a 0.2 µm membrane filter. While the mobile phase used for LC-MS consists of acidified water pH 3 (pH adjusted to 3 with formic acid) and acetonitrile with the ratio of (80: 20) for PER and (60:40) for AML respectively. The flow rate was 0.5 ml/min for UPLC method while for LC-MS were 0.250 and 0.2 ml/min for PER and AML respectively. Injection volume was 1.0 µl for UPLC method while for LC-MS was 10.0 µl. The detection for UPLC method was carried out at 210 nm while for the LC-MS the polarity was adjusted to be positive in case of AML and negative in case of PER.

Procedures

Stock solution preparation: Quantitatively transfer 20 mg of PER and 27.7 mg of AML into a 100 mL volumetric flask, add 75 ml of 0.01N HCl and dissolve by ultrasonic bath for 10 minutes, cool then complete to volume with the same solvent to obtain solution having a concentration equivalent to 200 µg mL^{-1} and 277 µg mL^{-1} respectively.

Working solution preparation: Dilute 5 ml from the stock solution preparation to a 25 ml volumetric flask then complete to the volume with the same solvent to obtain concentration equivalent to 40 µg $^{mL-1}$ and 55.4 µg mL^{-1} respectively.

Sample preparation: 10 tablets of Amloveran were weighed and finely powdered. Transfer quantity of powder equivalent to 20 mg PER and 27.7 mg AML to a 100 ml volumetric flask. Add 75 ml of solvent and sonicate for 15 minutes in room temperature then continue to volume with the same solvent. Filter through syringe filter 0.45 (discard not less than 5 ml) further, dilute 5 ml from this solution to 25 ml volumetric flask and complete to volume with the same solvent. Filter through a 0.45 µm membrane filter (Discard first 5 ml) and inject.

Validation: Validation items such as linearity, accuracy, limit of quantitation (LOQ), limit of detection (LOD), precision and specificity were performed according to ICH (44). Winks software was used in statistical analysis.

Linearity and construction of calibration graphs: Standard stock solutions of PER (200 µg /ml) and AML (277 µg /ml) were further diluted with the solvent (0.01N HCL) to obtain dilutions of PER and AML in the ranges of (20–64 µg /ml and 27.7–88.64 µg /ml), respectively. Two injections from each solution were injected (1.0 µL) and chromatographed. The calibration curve for each drug was obtained by plotting the average peak area for each solution against the corresponding concentrations.

Accuracy: Accuracy are typically established by preparing multiple samples containing the drug substance (PER and AML) and any other constituents present in the dosage form (e.g., excipients and coating materials) ranging in concentration from less than the lowest expected concentration to more than the highest concentration during release, three replicates at three levels (80%, 100% and 120%).

Precision

Repeatability: Repeatability assessed using a minimum of six determinations at 100 % of the test concentration.

Intermediate precision: Intermediate precision is assessed by using a minimum of six determinations at 100 % of the test concentration of the same homogenous sample by two analysts.

Quantification limit and detection limit: Limit of detection and quantification were carried out as per ICH guidelines. Limit of detection is considered as the minimum concentration with a signal to noise ratio of at least 3 while the limit of quantification is considered as a minimum concentration with a signal to noise ratio of at least 10.

Specificity

Forced degradation

For Perindopril Arginine: Forced degradation was carried out in 1N HCl, 1N NaOH and 3% H_2O_2 by transferring 20 mg of Perindopril Arginine to a 100 ml volumetric flask, 75 ml of 1N HCl, 1N NaOH or 3% H_2O_2 was added then dissolved by ultrasonic bath for 10 minutes and completed to volume with the same solvent. 5 ml from this solution was diluted to a 25 ml volumetric flask and completed to volume with mobile phase initially (without heating the stock) and after heating the stock solution at 70°C for 20, 40, 60 and 120 minutes.

For Amlodipine Besylate: Forced degradation was carried out in 1N HCl, 1N NaOH and 3% H_2O_2 by transferring 27.7 mg of Amlodipine Besylate to a 100 ml volumetric flask, 75 ml of 1N HCl, 1N NaOH or 3% H_2O_2 was added then dissolved by ultrasonic bath for 10 minutes and completed to volume with the same solvent. 5 ml from this solution was diluted to a 25 ml

volumetric flask and completed to volume with mobile phase initially (without heating the stock) and after heating the stock solution at 70°C for 20, 40, 60 and 120 minutes.

Placebo: The selected methods are chosen so as to selectively measure the active substance without any interference from other excipients in the dosage form.

Results and Discussion

A new RP-UPLC method for the analysis of the combination of PER and AML has been validated. The chromatographic conditions were adjusted so as to develop a stability indicating assay method and achieve high resolution not only between the two components but also the degradation products in the short run time. We tried different mobile phase composition but the best resolution was achieved when using a mobile phase consisting of Potassium phosphate buffer (pH 2.0) and acetonitrile with the ratio of (75:25%). The trials have proved that the pH of the mobile phase has a great effect on the peak shapes of the separated components. Selecting 254 nm wavelengths has shown optimum sensitivity for both drugs. Good separation with satisfactory resolution and run time were obtained using a flow rate of 0.5 mL/min. Under the previous optimized chromatographic conditions, PER, AML, and Degradation products were eluted and resolved.

Validation of the methods

Linearity and range: Linearity range, intercept (a), slope (b) and correlation coefficient (r) for the methods of analysis of PER and AML are listed in **TABLE 1.**

TABLE 1. **Methods of analysis of PER and AML**

Method parameters	**UPLC method**	
	Perindopril Arginine	**Amlodipine Besylate**
Wavelength	210	210
Linearity range	20 – 64 µg/ml	27.7 – 88.64 µg/ml
% Y-Intercept	-0.9776	-0.4481
Intercept (a)	-462.18	-707.21
Slope (b)	1491.83	3576.39
Correlation coefficient (r)	0.99985	0.99981
Accuracy (mean + RSD)		
80%	98.69 + 0.61	99.02 + 0.42
100%	98.97 + 0.73	99.40 + 0.97
120%	100.39 + 0.51	99.04 + 0.30
Precision **1-Intermediate Precision (mean + RSD)**		
Analyst 1 %RSD	99.04 + 0.310	99.86 + 0.389
Analyst 2 %RSD	99.33 + 0.518	99.32 + 0.686
2-Repeatability (mean + RSD)	98.96 + 0.452	100.07 + 0.477
LOD	1.0036	1.5831

LOQ	3.0414	4.7974

Limit of detection and limit of quantification: The results of LOQ and LOD for the methods of analysis of PER and AML are given in **TABLE 1**.

Accuracy: The accuracy of the developed method was validated by analyzing both drugs PER and AML in their matrix at three levels of the assayed amount: 80, 100 and 120%. The concentrations of both drugs were calculated. The results are shown in **TABLE 1**.

Precision: Intermediate precision was evaluated by calculating intra-day and inter-day precision. This was done by repeating the assay six times of the same concentration at 100% in the same day. Another six preparations were assayed in the different day by the different analyst, using the developed method and calculating relative standard deviation (RSD) for the results. The results of intermediate precision are shown in **TABLE 1**.

System suitability test: The results of peak system suitability for PER and AML in **TABLE 2**.

TABLE 2. **The results of peak system suitability for PER and AML.**

Parameters	**Results for PER**	**Results for AML**
Capacity factor K´	3.1	12.31
Tailing factor	1.13	1.14
Theoretical plate BP	5379	8691
Resolution	NA	23.44
% RSD, N= 6	0.419	0.456

Robustness: The method was successfully tested for robustness through a change of flow rate ± 0.1, temperature ± 1, wavelength ± 1, buffer pH ± 0.1and buffer percentage ± 1%. The results are in **TABLE 3** and **4** for PER and AML respectively. **FIG. 1.**

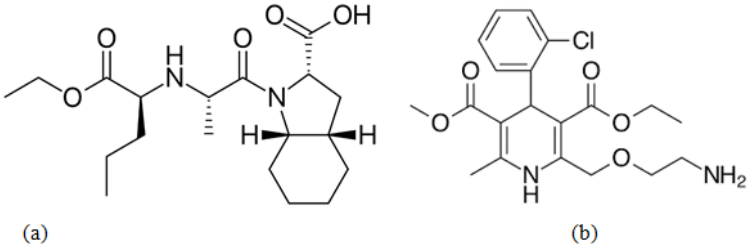

FIG. 1. **Chemical structures for (a) PER and (b) AML.**

TABLE 3. **For Perindopril Arginine.**

Perindopril Arginine	**Perindopril Arginine**		
	K prime	**Tailing factor**	**Plates**
Reference	3.1	1.13	5371
pH change to 1.9	3.09	1.13	5430
pH change to 2.1	3.09	1.13	5517
Temperature 44°C	3.2	1.12	5933
Temperature 46°C	3.16	1.1	5292
Wavelength 209 nm	3.1	1.14	5450
Wavelength 211 nm	3.14	1.13	5313
Buffer 74 %	2.79	1.14	6193
Buffer 76 %	3.27	1.11	4402
Flow rate 0.4 ml/min	4.35	1.07	6602
Flow rate 0.6 ml/min	2.49	1.09	4882

TABLE 4. **For Amlodipine Besylate.**

Amlodipine Besylate	**Amlodipine Besylate**			
	K prime	**Tailing factor**	**Plates**	**Resolution**
Reference	12.31	1.14	8684	23.43
pH change to 1.9	12.32	1.13	8811	23.64
pH change to 2.1	12.29	1.14	8770	23.6
Temperature 44 °C	12.41	1.12	8941	23.73
Temperature 46 °C	12.65	1.1	8658	23.56
Wavelength 209 nm	12.37	1.11	8429	23.34
Wavelength 211 nm	12.53	1.11	8328	23.23
Buffer 74 %	10.3	1.14	9266	22.91
Buffer 76 %	14.83	1.13	7953	24.18
Flow rate 0.4 ml/min	16.33	1.12	9433	24.81
Flow rate 0.6 ml/min	10.5	1.12	8091	22.77

Specificity

Forced degradation:

For Perindopril Arginine:

Acid Hydrolysis of drug substance: There was a decrease in Perindopril Arginine peak area (Degraded by 5.0%) without the appearance of any degradation product peak area.

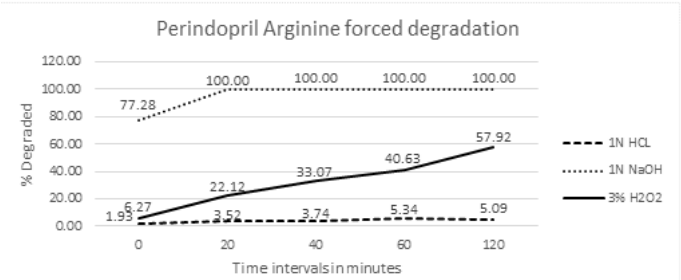

FIG. 2. **Perindopril Arginie forced degradation.**

FIG. 2. illustrate the percentage of degradation versus the time interval in minutes.

Base hydrolysis of drug substance: There was a decrease in Perindopril Arginine peak area (Degraded by 100.0%) with the appearance of one degradation product peak area at about 1.4 minutes.

Oxidation of drug substance: There was a decrease in Perindopril Arginine peak area (Degraded by 57.9%) with the appearance of one degradation product peak area at about 0.75 minutes.

For Amlodipine Besylate

Acid hydrolysis of drug substance: There was a decrease in Amlodipine Besylate area (Degraded by 100.0%) with the appearance of three degradation products peak area at about 1.7, 1.9 and 2.0 minutes.

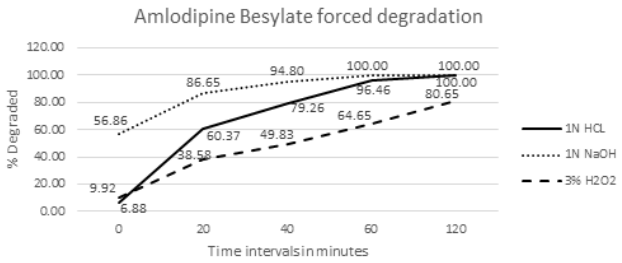

FIG. 3. **Amlodipine Besylate forced degradation**.

FIG. 3. illustrate the percentage of degradation versus the time interval in minutes.

Base hydrolysis of drug substance: There was a decrease in Amlodipine Besylate peak area (Degraded by 100.0%) with the appearance of three degradation products peak area at about 0.5, 0.7, 0.9 minutes.

Oxidation of drug substance: There was a decrease in Amlodipine Besylate peak area (Degraded by 80.6%) with the appearance of two degradation product peak area at about 1.7 and 2.0 minutes.

Placebo: The method is applied to a placebo, which is prepared as an authentic sample containing all ingredients except the active substance; the placebo is treated in exactly the same manner for assay considering the highest range of detection, The method showed no interference by the presence of any of the excipients.

Structure elucidation for the degradation products by LC-MS

Perindopril Arginine

Acid-induced degradation: Upon exposure Perindopril Arginine to 0.05N HCl for 2 hours without heating, the structure elucidation by LC/MS revealed the following possible pathways for the degradations (**FIG. 4(a)-4(d**)).

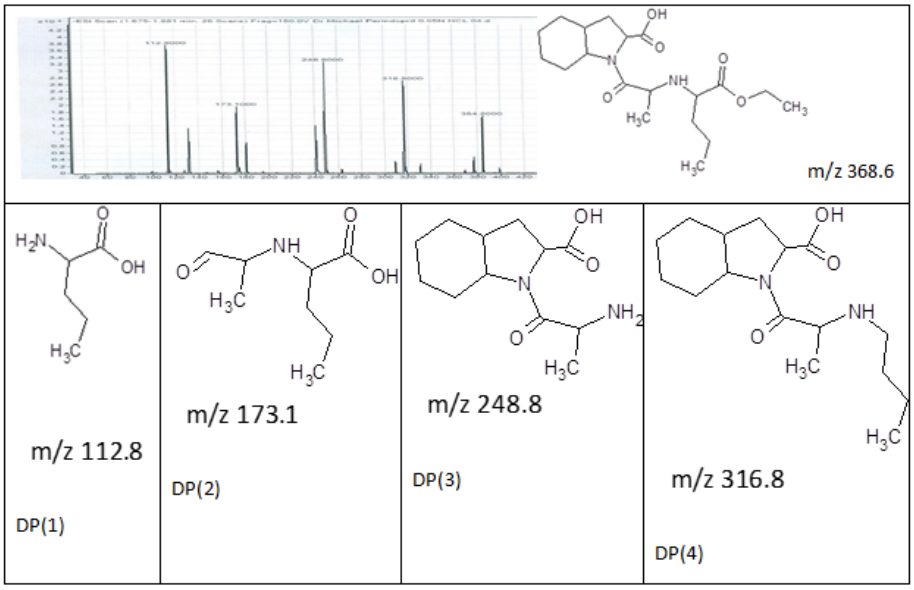

FIG. 4(a). **Possible degradation products for Perindopril Arginine in the Acid hydrolysis.**

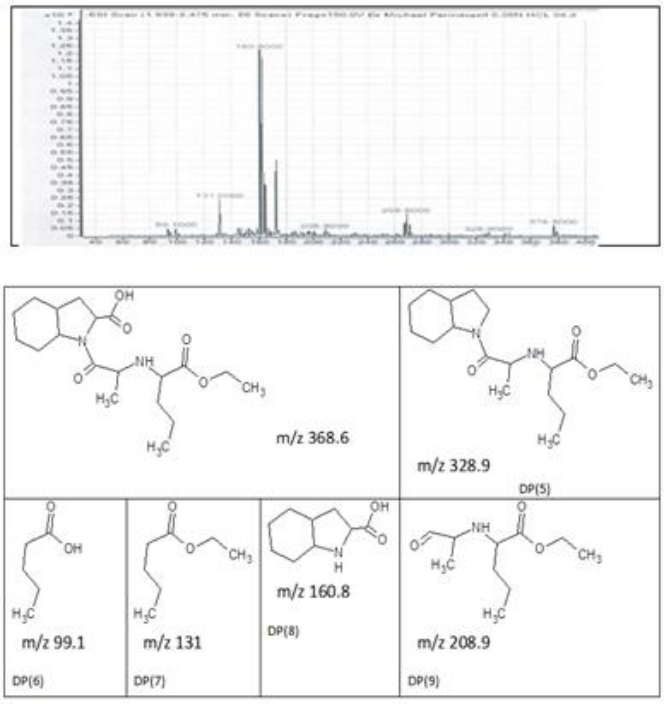

FIG. 4(b). **Possible degradation products for Perindopril Arginine in the Acid hydrolysis.**

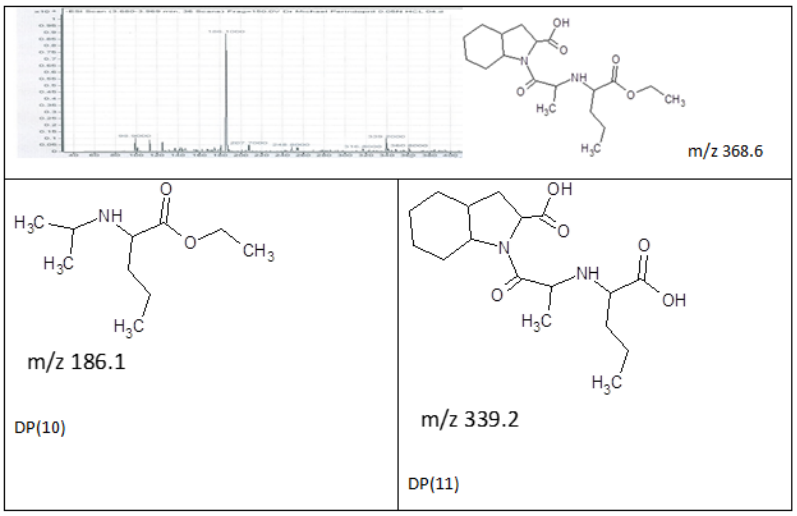

FIG. 4(c). **Possible degradation products for Perindopril Arginine in Acid hydrolysis.**

Alkaline-induced degradation: Upon exposure Perindopril Arginine to 0.05N NaOH for 2 hours without heating, the structure elucidation by LC/MS revealed the following possible pathways for the degradations (**FIG. 5(a)-(c)**).

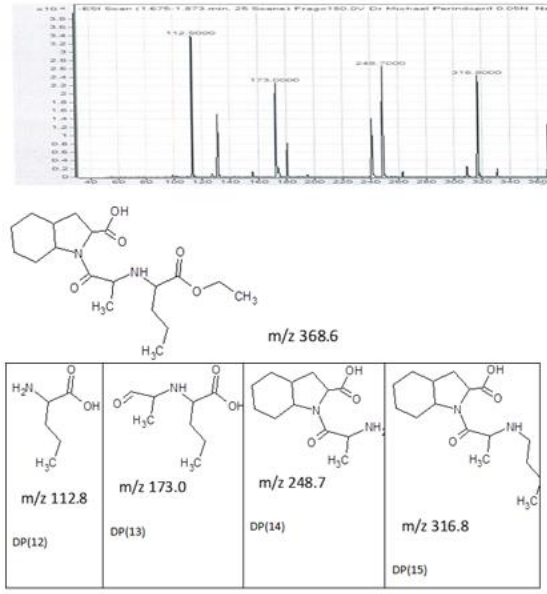

FIG. 5(a). **Possible degradation products for Perindopril Arginine in alkaline hydrolysis.**

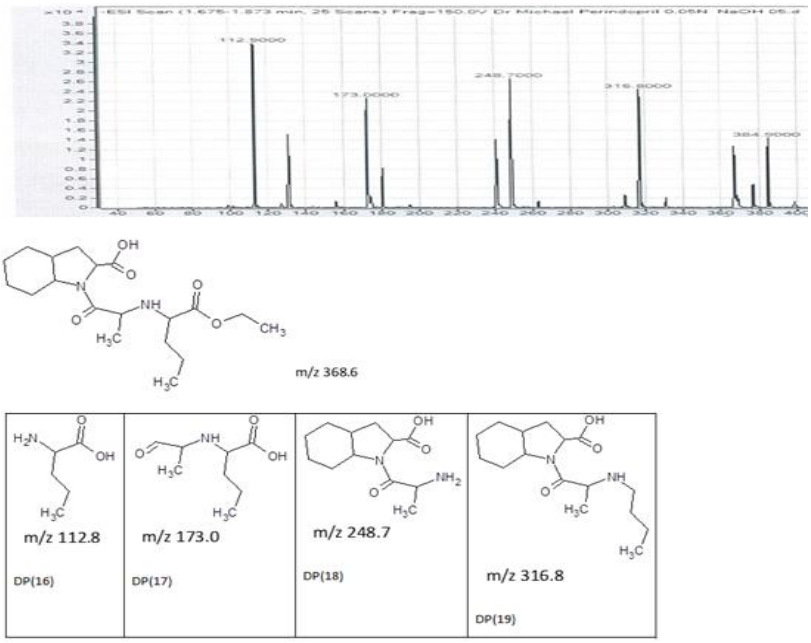

FIG. 5(b). **Possible degradation products for Perindopril Arginine in alkaline hydrolysis.**

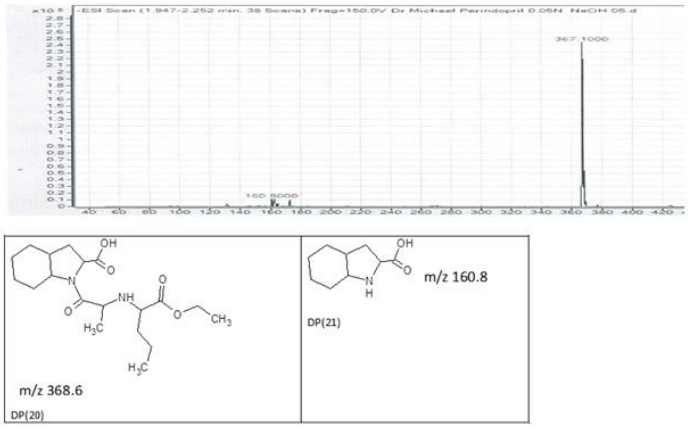

FIG. 5(c). **Possible degradation products for Perindopril Arginine in alkaline hydrolysis.**

Oxidation-induced degradation: Upon exposure Perindopril Arginine to 0.3% H_2O_2 for 2 hours without heating, the structure elucidation by LC/MS revealed the following possible pathways for the degradations (**FIG. 6(a)-6(b)**).

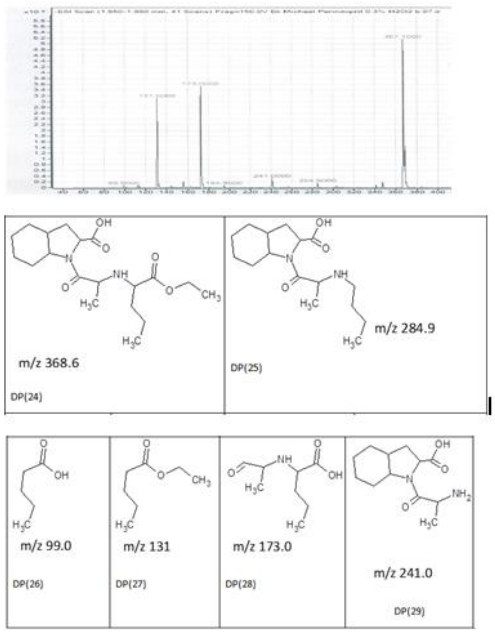

FIG. 6(a). **Possible degradation products for Perindopril Arginine in oxidative stress.**

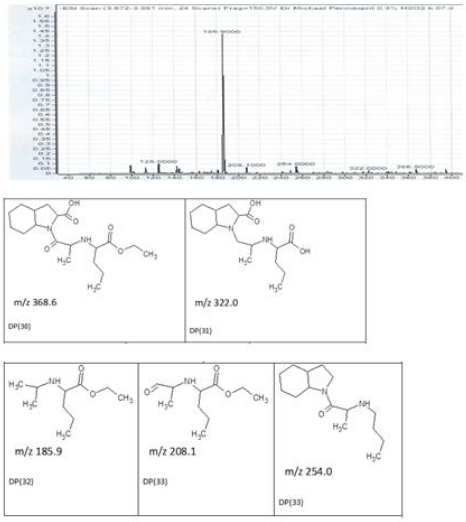

FIG. 6(b). **Possible degradation products for Perindopril Arginine in oxidative stress.**

Amlodipine Besylate

Acid-induced degradation: Upon exposure Amlodipine Besylate to 0.05N HCl for 2 hours without heating, the structure elucidation by LC/MS revealed the following possible pathways for the degradation (**FIG. 7(a)-7(d))**.

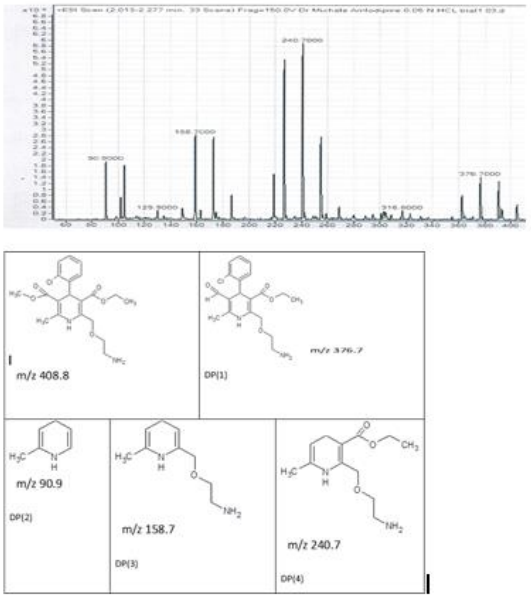

FIG. 7(a). **Possible degradation products for Amlodipine Besylate in acid hydrolysis.**

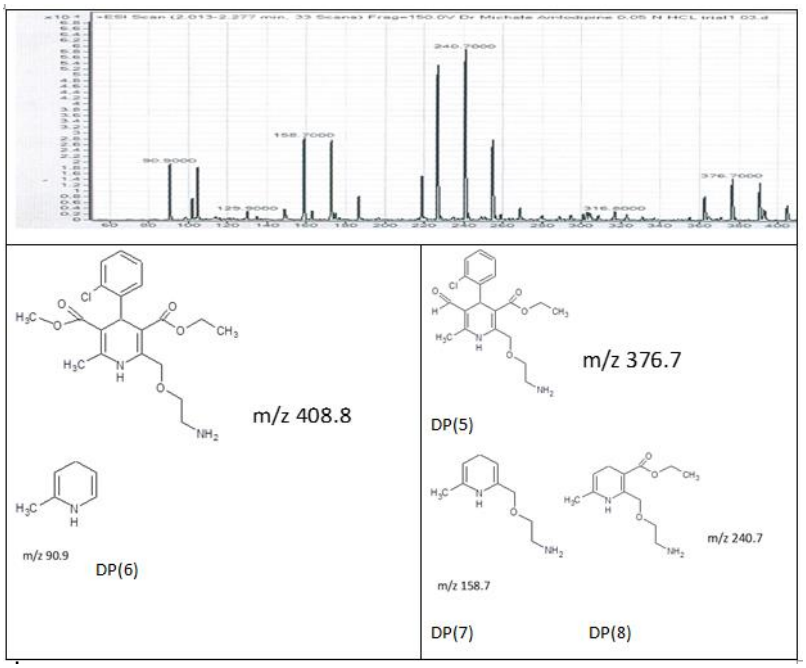

FIG. 7.(b) **Possible degradation products for Amlodipine Besylate in acid hydrolysis**

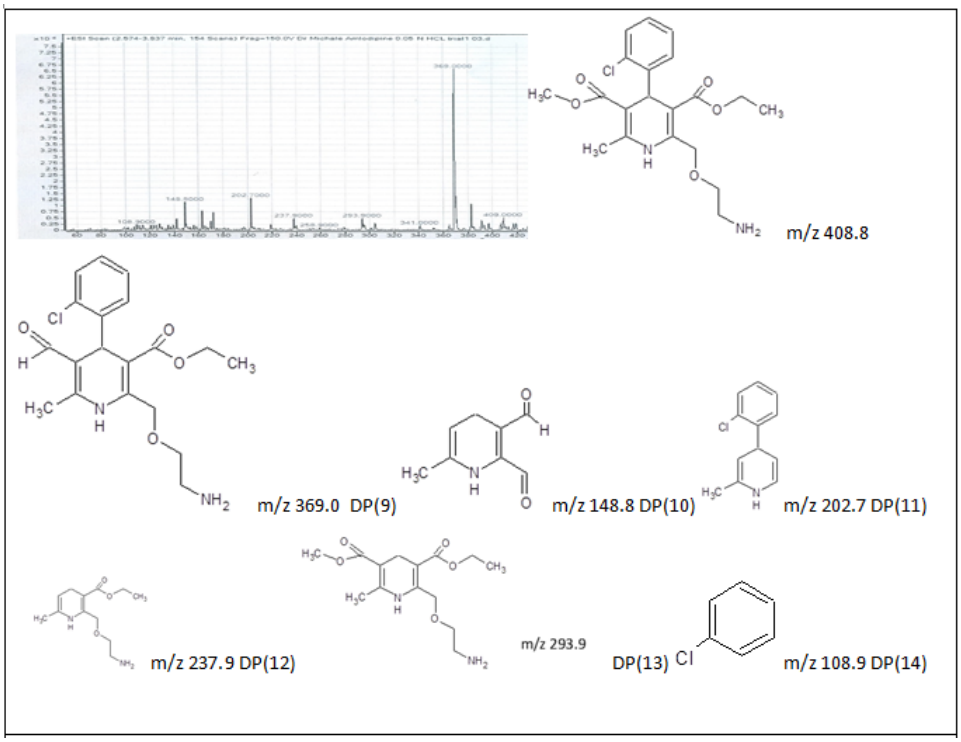

FIG. 7.(c) **Possible degradation products for Amlodipine Besylate in acid hydrolysis**

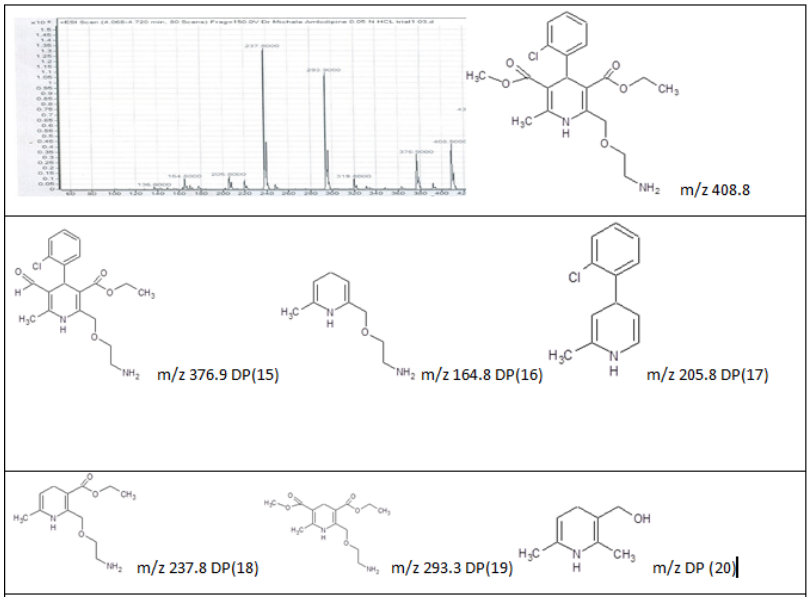

FIG. 7.(d) **Possible degradation products for Amlodipine Besylate in acid hydrolysis**

Alkaline-induced degradation: Upon exposure Amlodipine Besylate to 0.05N NaOH for 2 hours without heating, the structure elucidation by LC/MS revealed the following possible pathways for the degradation (**FIG. 8**).

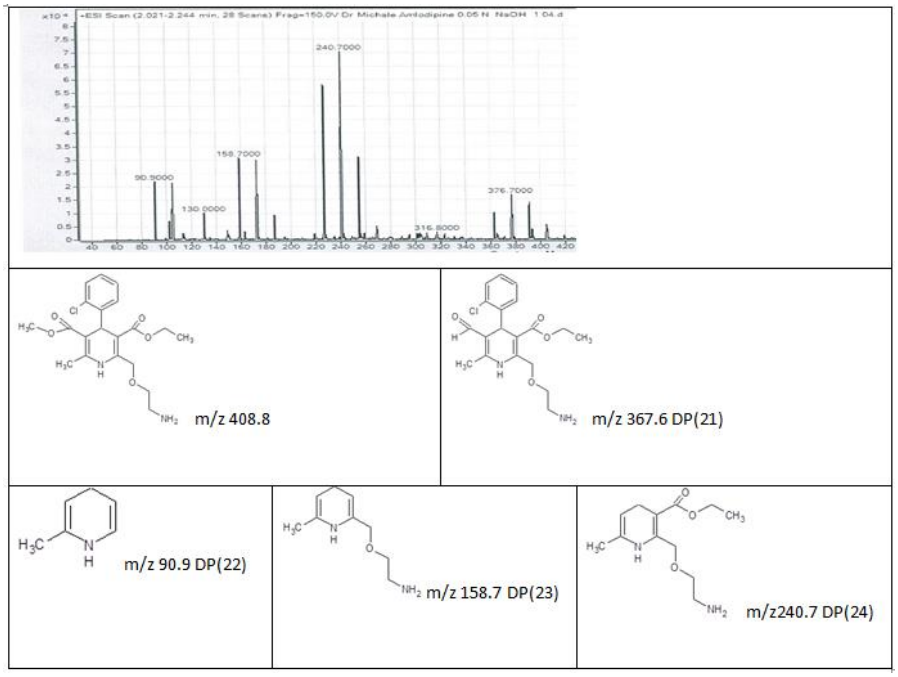

FIG. 8(a) **Possible degradation products for Amlodipine Besylate in alkaline hydrolysis.**

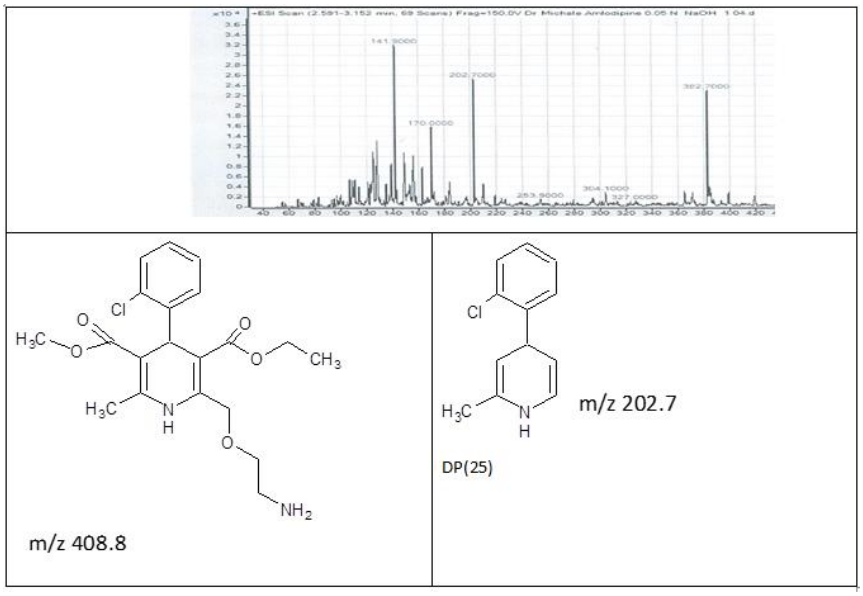

FIG. 8(b) **Possible degradation products for Amlodipine Besylate in alkaline hydrolysis.**

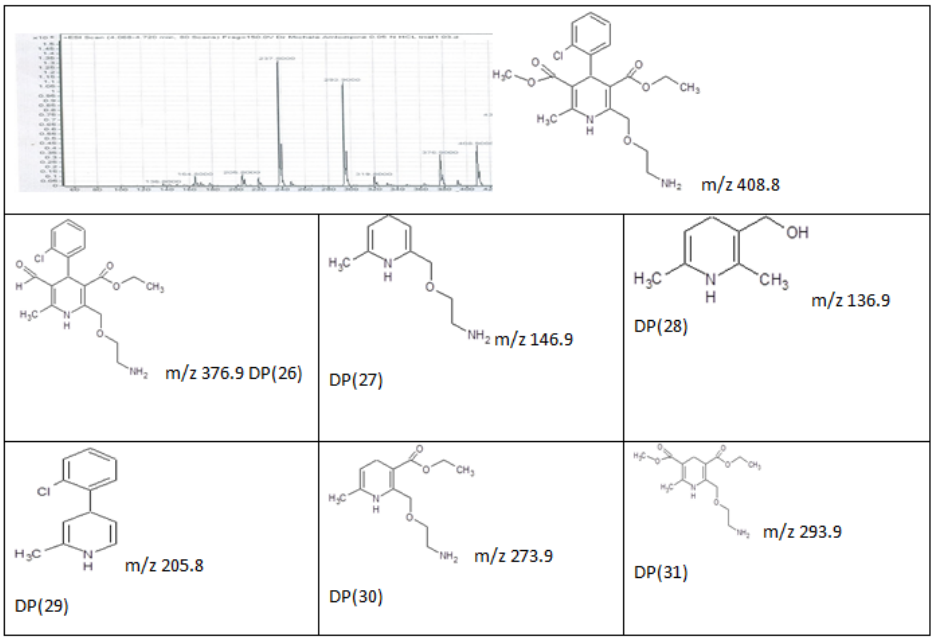

FIG. 8(c) **Possible degradation products for Amlodipine Besylate in alkaline hydrolysis.**

Oxidation-induced degradation: Upon exposure Amlodipine Besylate to 0.3% H_2O_2 for 2 hours without heating, the structure elucidation by LC/MS revealed the following possible pathways for the degradation (**FIG. 9**).

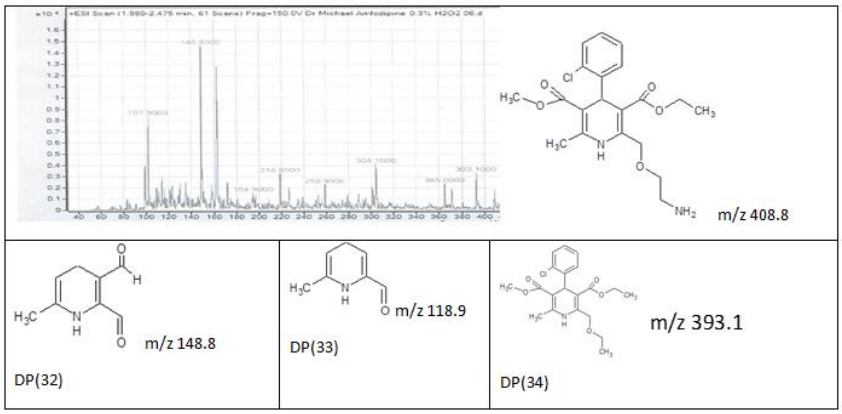

FIG. 9(a). **Possible degradation products for Amlodipine Besylate in oxidative stress.**

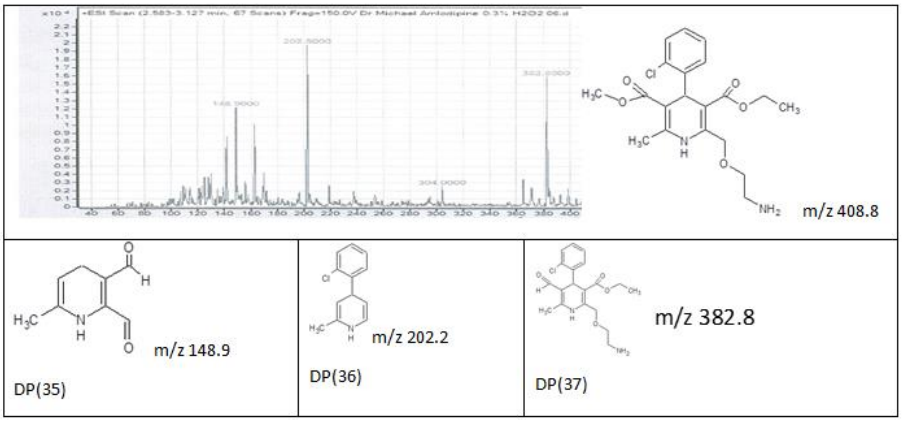

FIG. 9(b). **Possible degradation products for Amlodipine Besylate in oxidative stress.**

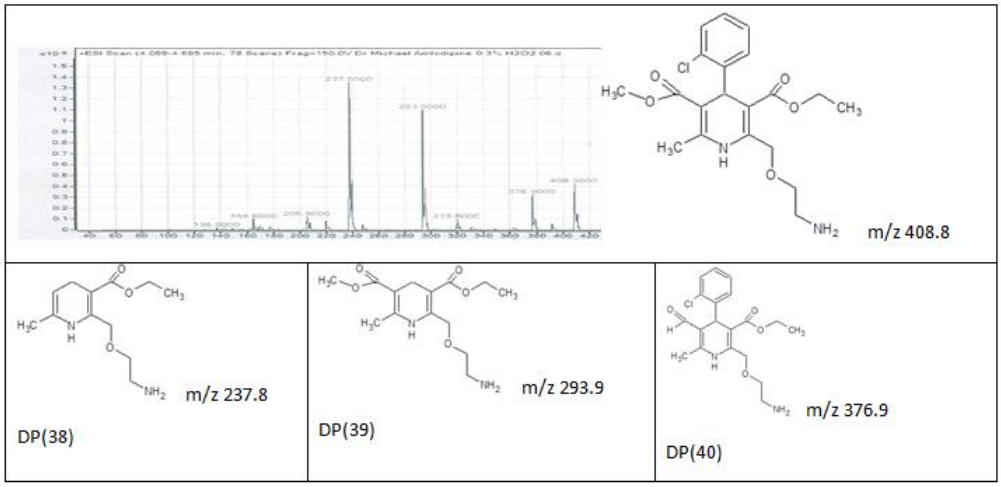

FIG. 9(c). **Possible degradation products for Amlodipine Besylate in oxidative stress.**

Discussion and Conclusion

The new stability indicating assay UPLC method was developed and validated for the determination of both PER and AML at the same time. The proposed method has many advantages over the reported method[43], regarding analysis time (4 minutes for UPLC method showed in **FIG. 10** versus 8 minutes for HPLC reported method), sensitivity (UPLC method is more sensitive than HPLC reported method where 1.0 µl and 20 µl were used as injection volumes respectively, Taking in consideration the reduced flow rate where it was 0.5 ml/min for UPLC method versus 1.0 ml/min for the reported method and of course cost saving. It is considered as a green chemistry and an environment friendly method due to chemical, materials and solvents savings appeared as short run time, very small injection volume and reduced flow rate. Also UPLC method showed very good system suitability especially in theoretical plates and resolution which were higher than that were obtained by the HPLC reported method (For reported method theoretical plates were 5113 and 4967 for PER and AML respectively while for the UPLC method they were 5379 and 8691 PER and AML respectively, on the other hand, resolution between

AML and PER were 2.19 and 23.44 for reported method and UPLC method respectively). UPLC robustness was done by changing pH, flow rate, mobile phase composition, wavelength and temperature with respect to system suitability parameters (capacity factor, theoretical plates, tailing factor and resolution) while in the robustness of HPLC reported method, the change in wavelength, flow rate and temperature were not performed, and the preformed items were done with respect to percentage recovery and RSD. The most important advantage over the reported method is that the method is considered as green chemistry method as due to the short run time and also is doesn't use tetrahydrofuran in mobile phase as the reported method. On the other hand, the reported method has an obvious problem in the baseline around the PER peak which may hinder in accurate integration. finally, the structure elucidation study for the degradation products of both actives (PER and AML) was done in a novel way to include most of the degradation products induced due to acidic, alkaline and oxidative degradations while the reported method took into consideration only one degradation pathway for PER and AML in alkaline medium only. The structure elucidation for PER by LC-MS has revealed that the common degradation products are resulting from the breakage of ester bond (**FIG 4**, degradation product 11) or amide bond (**FIG 4**, degradation product 3) or both (**FIG 4**, degradation product 1), on the other hand, the structure elucidation of the degradation products of AML has revealed that most of them are resulting from the breakage of ether bond (**FIG 8**, degradation product 21), losing the side chain (**FIG 8**, degradation product 25) or breaking the bond between the two cycles (**FIG 8**, degradation product 31).

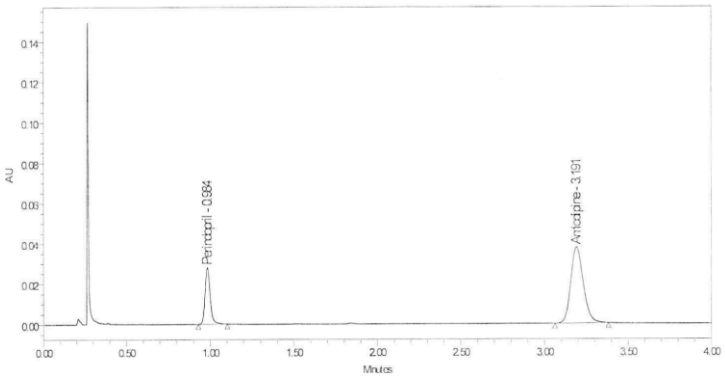

FIG. 10. **UPLC chromatogram for Perindopril Arginine and Amlodipine Besylate.**

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Declarations

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Ethical approval: Not required

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