International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 14, Issue 2, 2022

Original Article

ASSAY METHOD DEVELOPMENT AND VALIDATION OF CILNIDIPINE AND RAMIPRIL, CHARACTERIZATION OF ITS DEGRADANTS BY USING LC-MS/MS

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Received: 11 Nov 2021, Revised and Accepted: 03 Jan 2022

ABSTRACT

Objective: The current study focused on the development, validation of Ramipril and Cilnidipine using HPLC and characterization of forced degradation products using LC-MS/MS.

Methods: Simple, accurate, and precise method has been developed for the simultaneous estimation of Cilnidipine and Ramipril in tablet type of dosage form using X-bridge phenyl column (150x4.6 mm, 3.5m) with mobile phase of buffer and Acetonitrile in the 30:70 v/v ratio was pumped through a column with a 1 ml/min flow rate. The buffer used in this process was 0.1 percent tri ethyl amine in 1 lt of water and adjust its pH-2.5 with 0.1 percent ortho phosphoric acid. At ambient temperature, chromatography was isocratically performed and run time was 8 min. 240 nm was the optimized wave length.

Results: Cilnidipine and Ramipril retention times were 2.79 min, 5.11 min. respectively. By injecting the norm six times, device parameters of suitability have been studied and the results were found to be well under the acceptance criteria. This approach offers strong linearity over a range of 2-30 µg/ml Cilnidipine 1-15 µg/ml Ramipril concentrations. The regression coefficient R^2>0.999 from the calibrated curve implies that the linearity of the system was within the range.

Conclusion: The system was validated by using HPLC in terms of accuracy, linearity, method precision, accuracy, limit of detection, limit of quantification, robustness, degradation and the degradation products were characterized by using LC-MS/MS.

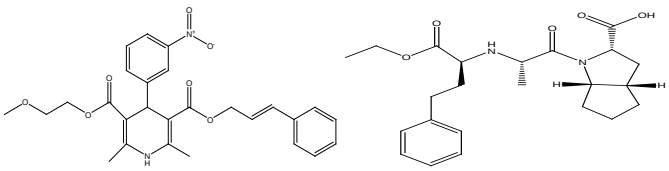
Keywords: Cilnidipine, Ramipril, Development, Validation, RP-HPLC

DOI: https://dx.doi.org/10.22159/ijap.2022v14i2.43570. Journal homepage: https://innovareacademics.in/journals/index.php/ijap

INTRODUCTION

Cilnidipine is a calcium channel blocker [1]. Cilnidipine is approved to treat hypertension [2]. Accompanied by L-type calcium channel blocking [3] functions, it is a calcium antagonist. Cilnidipine in addition on the L-type calcium channel, unlike other calcium antagonists, it can operate on the N-type calcium channel Cilnidipine reduces blood pressure and is used for the prevention and comorbidity of hypertension [4, 5] Cilnidipine dilates both arterioles and venues due to its blocking action at the L-type and N-type calcium channels, thus reducing the pressure in the capillary bed. Cilnidipine has a mild direct dromotropic effect, effective vasodepressor and arrhythmia-inhibiting effect [6, 7] and vasoselective effect.

Ramipril is a medication used to treat blood pressure, heart failure [8, 9], and kidney disease with diabetes [10, 11] also used to prevent cardiovascular disease [12, 13]. It is fair initial care and it is swallowed by mouth. Headaches [14], dizziness [15, 16], tiredness and cough [17] are typical side effects. Liver complications [18], angioedema [19, 20], kidney problems, and high blood potassium [21, 22]. Use in pregnancy and breastfeeding is not recommended. It is an ACE inhibitor [23] and works by decreasing renin-angiotensin-aldosterone [24] system activity. Fig. 1 shows the chemical structures of Ramipril and Cilnidipine. The aim of the study is to separate the pharma ingredients Ramipril and Cilnidipine by using RP-HPLC and characterization of its degradants using LC-MS/MS.



Cilnidipine **Ramipril**

Fig. 1: Chemical structures of cilnidipine and ramipril

MATERIALS AND METHODS

Chemicals

Acetonitrile, tri ethylamine, orthophosphoric acid and water (HPLC Grade) were bought from Merck India Ltd, Worli, Bombay, India. All APIs of Cilnidipine, ramipril were bought from Glenmark pharmaceutical private Ltd, Andheri, Mumbai, India, were procured as reference levels.

Equipment

HPLC

The chromatographic device of Waters e-2695 quaternary pump, PDA detector of 2998 and chromatographic software Empower 2.0 was used.

LC-MS/MS

An HPLC system (waters alliance e2695 model) connected with mass spectrometer [25] QTRAP 5500 triple quadrupole instrument (sciex) was used [26-28].

Chromatographic conditions

Chromatographic separation of drugs Cilnidipine and Ramipril has been carried out in isocratic mode at room temperature using waters X-bridge phenyl column (150x4.6 mm, 3.5 µm). The mixture of 0.1% TEA pH-2.5 adjusted with OPA: acetonitrile in 30:70 v/v with a flow of 1 ml/min was used as the mobile process. The injection volume was 10 µl and at 240 nm the eluents were controlled with a running time of 8 min.

Preparation of buffer

1 ml of tri ethyl amine was transferred into 1 lt of HPLC water and mixed well; adjust the pH-2.5 with OPA. Filtered and degassed [29] through 0.45 µ membrane filter paper.

Selection of mobile phase

Prepared a buffer and Acetonitrile combination (30:70v/v). The selected mobile phase has given sharp peaks with low tailing factor.

Selection of wavelength

The absorption spectra of solution of each Cilnidipine and Ramipril were scanned over a range of 200-400 nm by using spectrophotometer and the spectra were recorded. Two drugs were absorbed maximum at a wavelength of 240 nm.

Preparation of diluent

The mobile phase was used as diluent.

Standard solution preparation

Weighed 20 mg Cilnidipine, 10 mg of Ramipril and transferred into 100 ml volumetric flask. 70 ml diluents was added and sonicated to dissolve for 15 min and made up to the mark with diluents. 5 ml of the above solution was further diluted to 50 ml with diluents.

Validation procedure

As per the guidelines of ICH Q2 (R1) analytical method was validated [30-34] in terms of parameters such as system suitability, method precision, specificity, intermediate precision, linearity, robustness, LOD, LOQ, and durability of forced degradation.

Suitability

To check the performance of the system, system suitability parameters were calculated. The parameters include the count of USP plates, USP tailing [35] and % RSD can be calculated and found to be within the limit.

Specificity

Specificity is the ability to assess unequivocally in the presence of an analyte, other impurities, and elements such as, expedients which may be assumed to be available in the sample and standard solution. It was checked by examining chromatograms of blank standard and the standard spiked [36] with Cilnidipine and Ramipril.

Accuracy

Accuracy is the closeness of the test result to the true value. The therapy was assessed by the study at three different concentration levels. A minimum of three injections have been given at each stage, the percentage recovery and the corresponding standard deviation, and the RSD have been determined.

Precision

The degree of agreement among individual tests results is the precision of an analytical technique. In terms of repeatability, intra-day and intra-day variations, the precision of the present method was evaluated. Analysis of the standards at different time intervals of the same day and on different days was carried out.

Linearity

The Linearity of an analytical method is its ability to obtain result is directly proportional to the analytes concentration. For the evaluation of the linearity range, seven series of standard solution were selected. Using peak area versus concentration of the standard solution, the calibration curve was plotted and the regression equations were measured using the method with the least squares.

LOD and LOQ

LOD is the lowest analyte quantity in the sample that can be detected, while LOQ is the lowest analyte quantity in the sample that can be determined with appropriate accuracy and precision. Based on the calibration curves, LOD and LOQ were separately determined. The LOD and LOQ were calculated according to ICH guidelines as 3.3σ/s and 10σ/s, respectively, where σ/s indicate the ratio of signal to noise.

Robustness

Analytical procedures robustness is a measure of its ability to remain unaffected by small but deliberate variations in parameters of the method and provides an indication of its reliability during normal use. The robustness study was carried out by injecting the standard solution into the HPLC system and modifying the flow rate (±0.2 ml/min), organic phase (±10 percent) of chromatographic conditions. The separation factor and retention time and maximum asymmetry have been measured.

Degradation

Stress degradation should be no interference between the peaks obtained for the forced degradation chromatogram. According to ICH guidelines [37], stress degradation studies were conducted. The peaks of degradation should be well separated and the resolution between the peaks should be at least 1.0 and the peak purity of the principal peaks [38] should pass.

RESULTS AND DISCUSSION

Method development and optimization

Initially, the RP-HPLC system was optimized using an X-Bridge phenyl (150x4.6 mm, 3.5 µm) column. To satisfy the system suitability parameters, a mixture of acetonitrile and TEA (70:30v/v) as the mobile phase (Flow rate 1.0 ml/min) was found to be more appropriate. Table 1 summarizes the optimized chromatographic conditions.

Table 1: Optimized chromatographic conditions

LC conditions		
Stationary phase	:	X-Bridge phenyl column (150x4.6 mm, 3.5 µm)
Mobile Phase	:	Acetonitrile and 0.1% TEA of pH-2.5 (70:30)
Elution mode	:	Isocratic Acetonitrile: Buffer = 70:30 % v/v
Flow rate	:	1.0 ml/min,
Sample volume	:	10µl using Rheodyne 7725i injector
Oven Temperature	:	Ambient
MS conditions		
Interface	:	ESI
Operation mode	:	MRM
Polarity	:	Positive
Capillary voltage	:	4 KV
Fragmentor voltage	:	170 V
Skimmer voltage	:	65 V
Nebulizer Gas flow	:	40 psig
Drying gas	:	10 L/min
Gasoline temperature	:	325 °C
Detection	:	m/z: 0-800
Data station	:	ABSCIEX

Tests for method validation

System suitability

To get stable baseline, the HPLC system was stabilized for 60 min. six replicate injections mixture containing 20 µg/ml of Cilnidipine and 10 µg/ml of Ramipril were performed and assessed to check the suitability of the system. From six replication injections, the system suitability parameters were evaluated. The research concludes that the system suitability and outcomes of the HPLC system being used are summarized below in table 2 [39].

Specificity

There was no interference [40] from the blank during the retention time of Cilnidipine and Ramipril. The process is, therefore, specific.

Table 2: Results of system suitability

System suitability parameter	Acceptance criteria	Cilnidipine			Ramipril		
		Mean	Std Dev	%RSD	Mean	Std Dev	%RSD
Retention time	NLT 2.0	2.79	0.010	0.35	5.129	0.007	0.13
USP plate count	NLT 2000	3741	39.484	1.06	9162	49.918	0.54
USP Tailing	NMT 2.0	1.05	0.005	0.52	0.97	0.033	1.39
Resolution	NLT 2.0	-	-	-	11.72	0.054	0.46

n=6

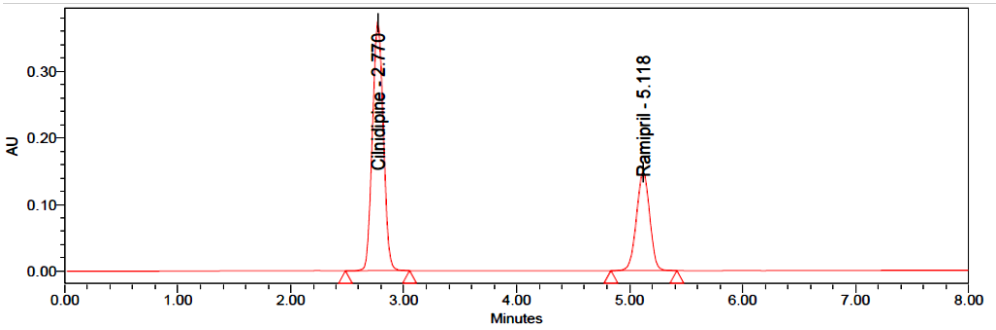


Fig. 2: Chromatogram of system suitability

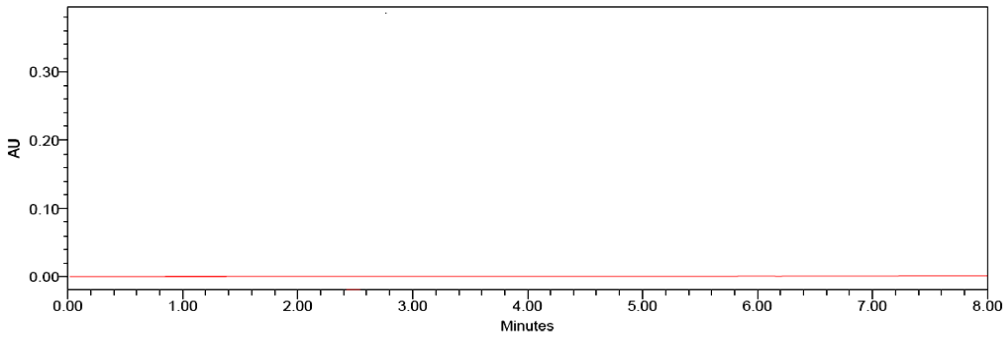


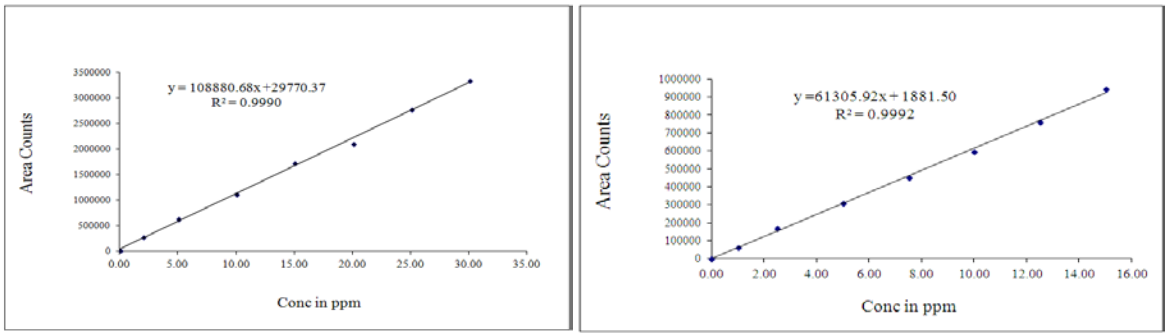
Fig. 3: Chromatogram of blank

Linearity

Linearity was calculated by plotting a calibration curve of peak area versus respective concentrations. From this calibration curve it was found that the curve was linear in the concentration range of 2-30 µg/ml of Cilnidipine and 1-15 µg/ml of Ramipril. The regression equations for Cilnidipine were Y=108880.68x+29770.37, and for Ramipril was Y=61305.92x+1881.5, respectively.

Table 3: Linearity data

Linearity level	Cilnidipine			Ramipril		
	Conc. (µg/ml)	Mean area counts	Std dev	Conc. (µg/ml)	Mean area counts	Std dev
Linearity-1	2	256890	238.854	1	62716	22.942
Linearity-2	5	621356	469.667	2.5	171818	259.485
Linearity-3	10	1105500	916.652	5	308822	15.308
Linearity-4	15	1711265	707.749	7.5	453374	963.735
Linearity-5	20	2096456	600.635	10	595546	281.896
Linearity-6	25	2771404	521.419	12.5	760828	254.772
Linearity-7	30	3325525	223.467	15	941815	300.435
Slope	108880.68			61305.92		
Intercept	29770.37			1881.5		
% RSD	0.999			0.9992		



A **B**

Fig. 4: Linearity plot of (A) Cilnidipine and (B) Ramipril

Accuracy

The accuracy of the method was performed by calculating the recovery experiments at three levels (50 percent, 100 percent 150 percent). APIs were prepared at concentrations of 10, 20, and 30 µg/ml of Cilnidipine and 5, 10, 15 µg/ml of Ramipril, respectively. For each spike level, the test solution was injected three times and an assay was performed as per the test method. The recovery results were close to 100 percent and the RSD values were less than 2 percent. Recovery values showed that, within the desired range, the method was accurate. The results were summarized below in table 4 [41].

Precision

In terms of intra-day and inter-day differences, the accuracy of this method was assessed. Six repeated analyses of the standard solution of Cilnidipine and Ramipril on the same day under the same experimental conditions were used to determine intraday studies. In the same laboratory, the intermediate precision of the method was carried out by studying the analysis with different analysts with different instruments. As the each concentration level, good recovery values were obtained, indicating that the procedure was accurate.

Table 4: Results of accuracy

Accuracy level	Cilnidipine		Ramipril	
	%Recovery	Std dev	%Recovery	Std dev
50%	100.1	0.294	98.8	0.525
100%	99.7	0.401	99.8	0.243
150%	99.8	0.071	99.4	0.365

n=3

Table 5: Results of precision

Parameter	Cilnidipine			Ramipril		
	Mean % recovery	Std dev	Conc. (µg/ml)	Mean % recovery	Std dev	Conc. (µg/ml)
Method precision	99.7	0.231	20	100.3	0.153	10
Intermediate precision	99.1	0.782	20	100.4	0.451	10

n=6

LOD and LOQ

Using the calibration curve process, LOD and LOQ were calculated separately. By injecting a progressively lower concentration of standard solution using the established RP-HPLC process, the LOD and LOQ of the compounds were calculated. The LOD values of Cilnidipine and Ramipril were 0.6µg/ml, 0.3µg/ml and s/n values were 6, 3 respectively. Cilnidipine and Ramipril LOQ values were 2.0 µg/ml, 1.0 µg/ml s/n values were 26, 24 respectively.

Robustness

As per ICH norms, small but intentional differences were made in the parameters of the method, for instance, like the change in the rate of flow (±0.2 ml/min) and organic phase (±10%) to check the method capacity to remain unaffected [42].

Table 6: Results of robustness

Change in parameter	Cilnidipine (% RSD)	Ramipril (% RSD)
Flow plus (1.2 ml/min	0.26	0.17
Flow minus (0.8 ml/min)	0.11	0.49
Organic plus (77:23)	0.37	0.24
Organic minus (63:37)	0.45	0.36

RSD-Relative standard deviation; All the values are presented as Mean (n=3)

Forced degradation

The proposed analytical method can be used for release and stability studies [43] for successful evaluations for and can be considered as a stability-indicating method. The survey of forced degradation [44] was conducted according to ICH guidelines includes acid, base, peroxide, reduction, thermal and hydrolysis degradation [45-47]. From the chromatograms, it is evident that selected drugs were stable under the applied stress conditions though the degradation peaks were obtained. The degradation samples were characterized by using LCMS.

Acid degradation

Acid degradation of Cilnidipine and Ramipril was studied in 1N HCl. 15.1% of Cilnidipine and 15.6% of Ramipril degradation was observed in HPLC, two degradation peaks of D_1 and D_4 were formed.

Alkali degradation

Alkali degradation of Cilnidipine and Ramipril was studied in 1N NaOH. 14.9% of Cilnidipine and 14.4% of Ramipril degradation were observed in HPLC, two degradation peaks of D_1 and D_4 were formed.

Peroxide degradation

Peroxide degradation of Cilnidipine and Ramipril was studied in 30% hydrogen peroxide. 13.2% of Cilnidipine and 11.3% of Ramipril

degradation was observed in HPLC, two degradation peaks of D_2 and D_5 were formed.

Reduction degradation

Reduction degradation of Cilnidipine and Ramipril were studied in 30% sodium bisulphate solution. 12.8% of Cilnidipine and 11.7% of Ramipril degradation was observed in HPLC, two degradation peaks of D_3 and D_6 were formed.

Thermal degradation

In thermal degradation sample was exposed to 105 °C for 6 h, 1.4% Cilnidipine and 2.3% Ramipril degradation was observed in HPLC. No degradation products were formed.

Hydrolysis degradation

Hydrolysis degradation of Cilnidipine and ramipril was observed in HPLC water. 1.2% Cilnidipine and 0.8% Ramipril degradation was observed. No degradation products were formed.

MS/MS degradation product

Fig. 6 shows the fragmentation mechanism of degradation product 1 of m/z-554, which was observed under conditions of acid and alkali degradation. Abundant productions are seen on the spectrum at m/z-432 (loss of $C_6H_4NO_2$ from m/z-554), m/z-390 (loss of $C_2H_2O_2C1_3$ from m/z-554), m/z-269 (loss of $C_6H_4NO_2$ from m/z-390), m/z-192 (loss of C_6H_5 from m/z-269) and m/z-108(loss of $C_4H_4O_2$ from m/z-192). The proposed structures were confirmed by the MS/MS experiments in combination with precise mass measurements.

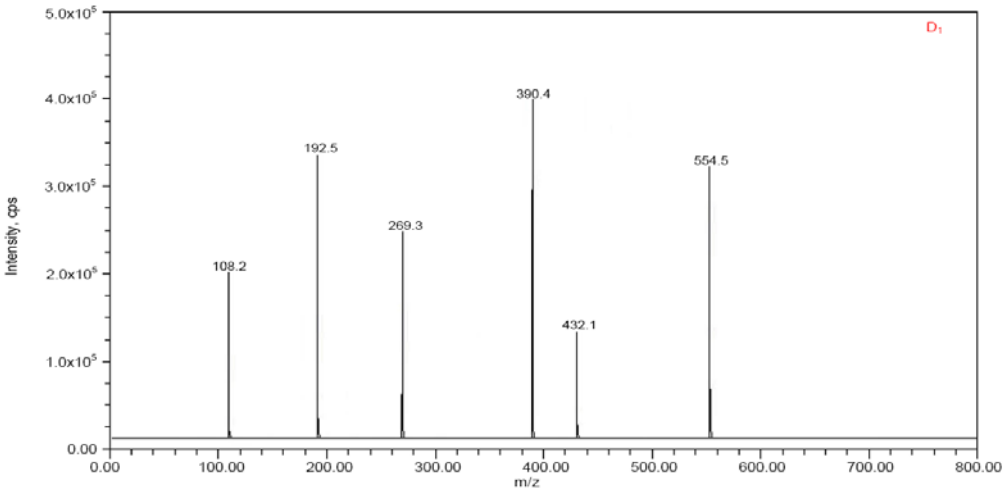
MS/MS degradation product

Fig. 7 shows the degradation product 2 fragmentation mechanism of m/z-434 observed under the condition of peroxide degradation. Abundant productions are seen on the spectrum at m/z-331 (loss of $C_4H_8O_3$ from m/z-434), m/z-228 (loss of $C_4H_8O_3$ from m/z-331), m/z-107 (loss of $C_6H_4NO_2$ from m/z-228), m/z-312 (loss of $C_6H_4NO_2$ from m/z-434). The proposed structures were confirmed by the MS/MS experiments in combined with accurate mass measurements.

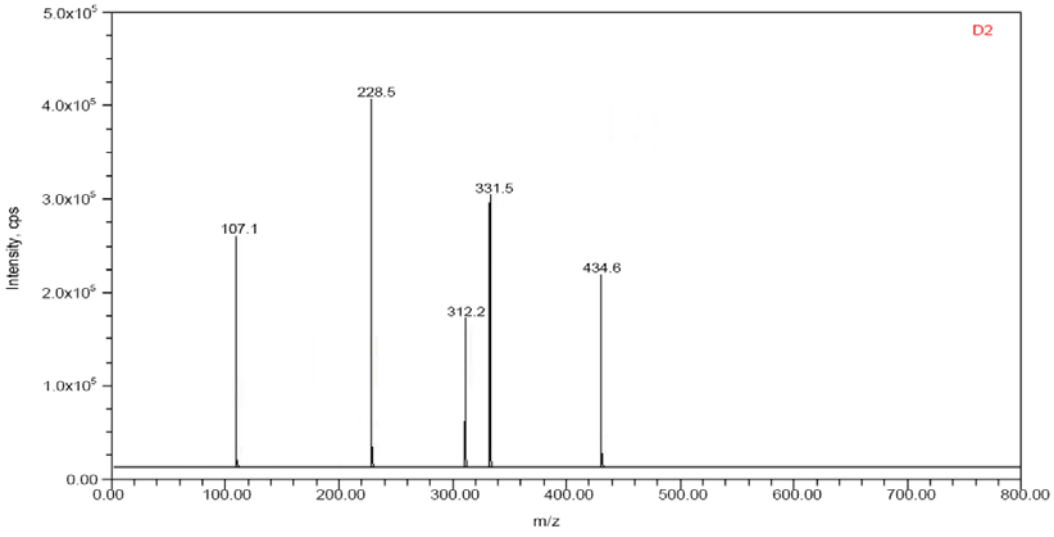
Table 5: Results of forced degradation

Stress condition	Cilnidipine % degradation	Ramipril % degradation
Acid degradation	15.1	15.6
Alkali degradation	14.9	14.4
Peroxide degradation	13.2	11.3
Reduction degradation	12.8	11.7
Thermal degradation	1.4	2.3
Hydrolysis degradation	1.2	0.8

Data expressed as mean, (n=3)



A



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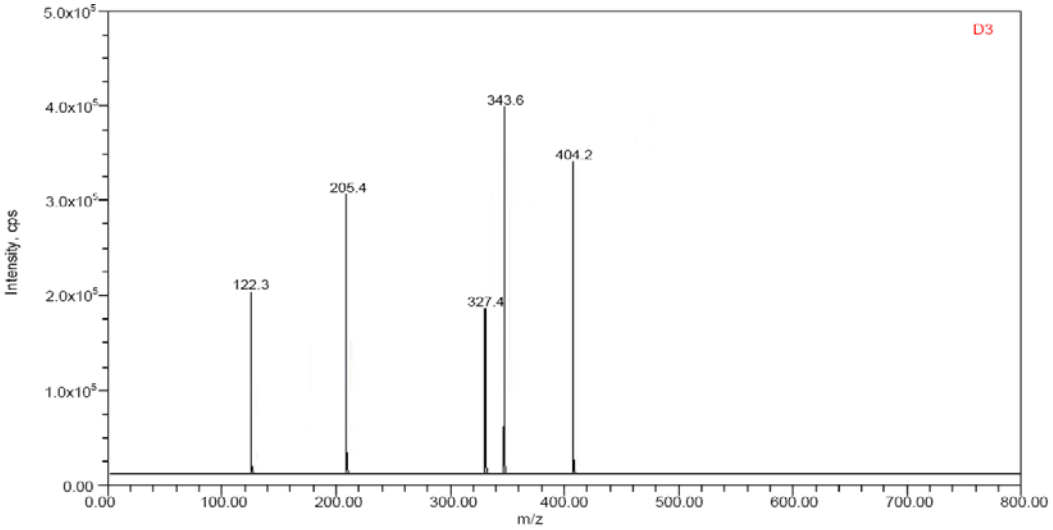
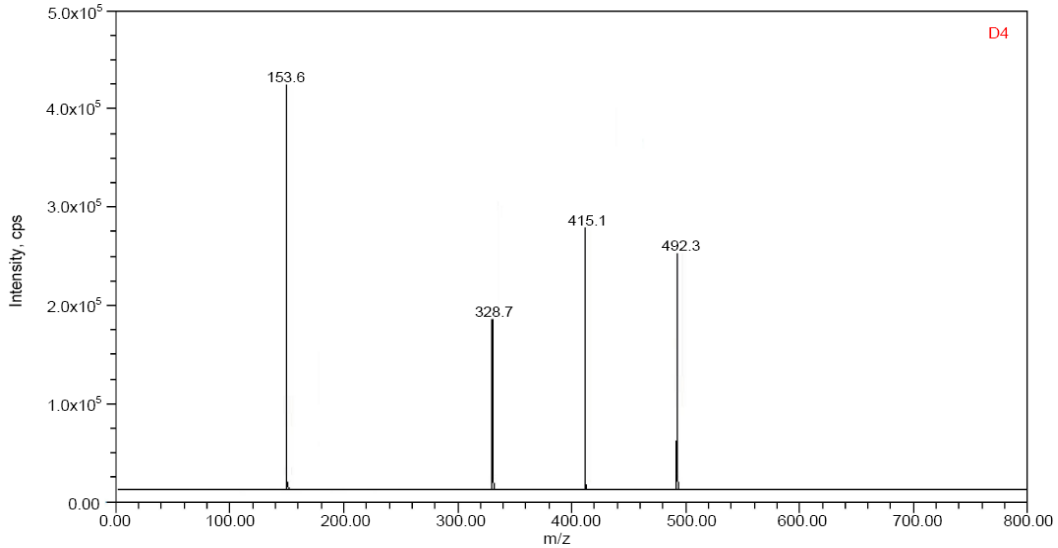
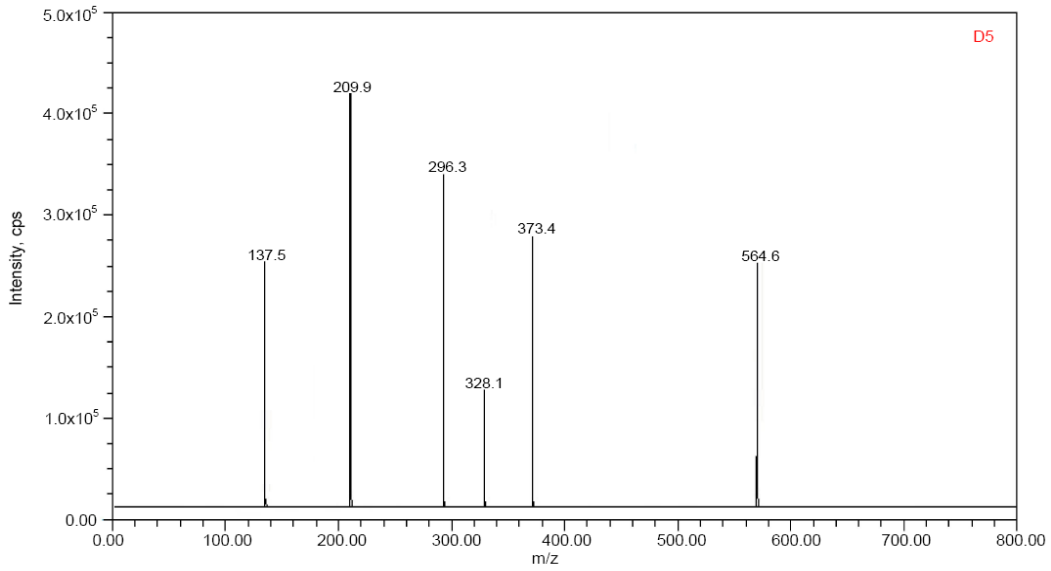

D3
343.6
404.2
205.4
122.3
327.4
Intensity, cps
m/z

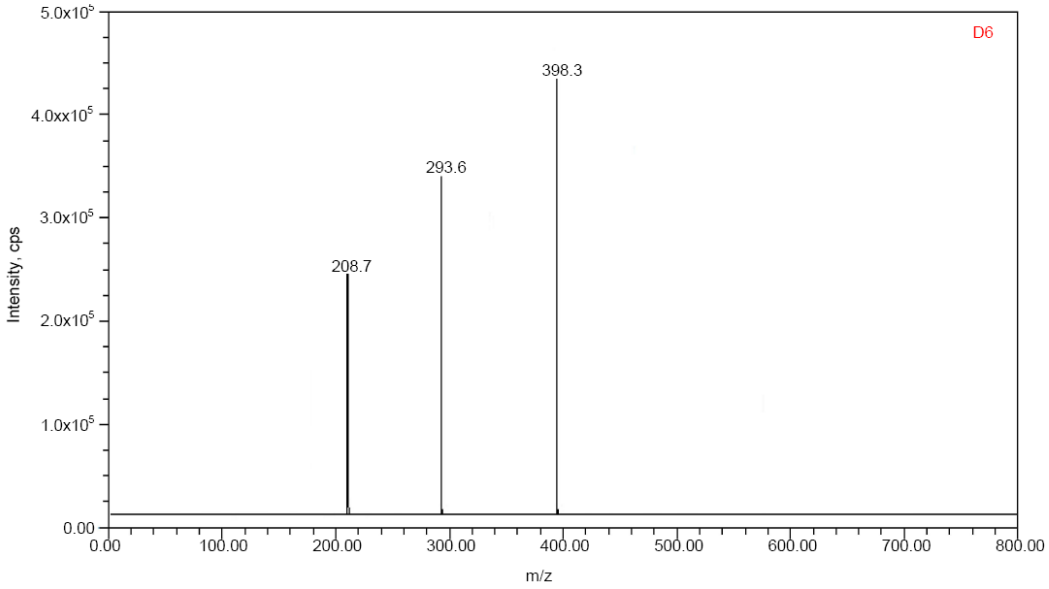
C


D4
153.6
415.1
492.3
328.7
Intensity, cps
m/z

D


D5
209.9
296.3
373.4
137.5
564.6
328.1
Intensity, cps
m/z

E



F

Fig. 5: Mass spectras of (A) D_1 (B) D_2 (C) D_3 (D) D_4 (E) D_5 and (F) D_6

Collision induced dissociation of cilnidipine and ramipril

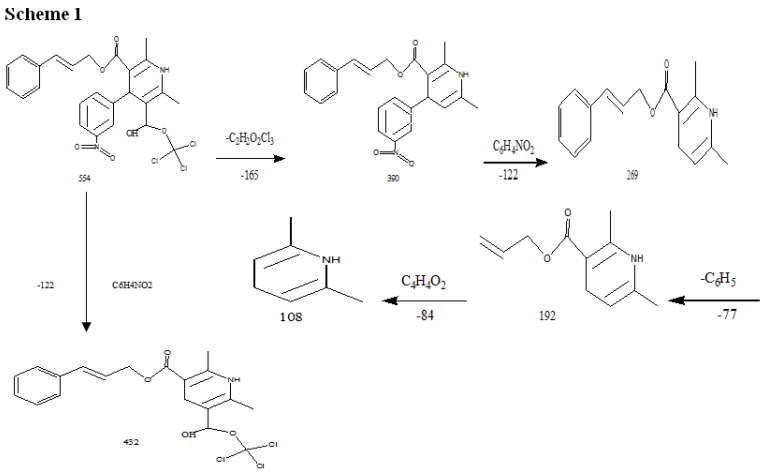


Fig. 6: Mechanism for proposed fragmentation of DP_1 (m/z-554)

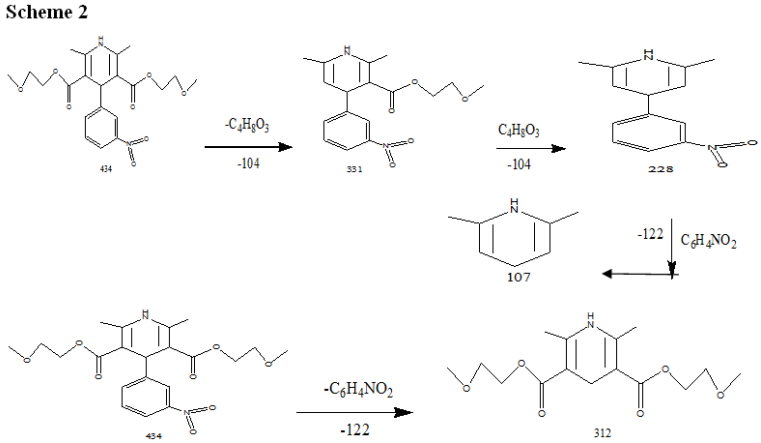


Fig. 7: Proposed fragmentation mechanism of DP_2 (m/z-434)

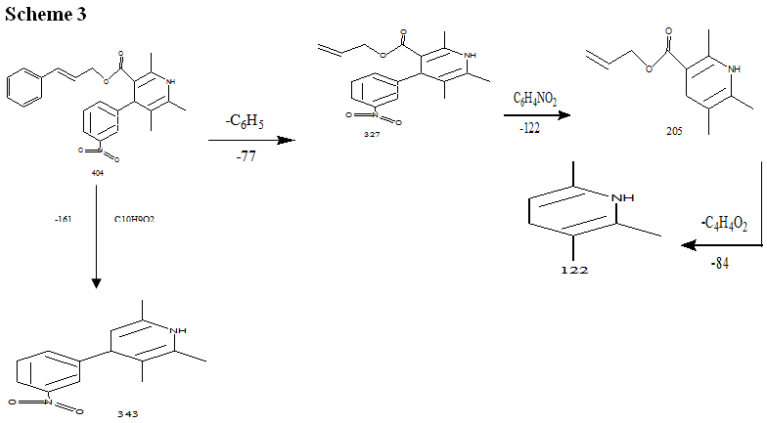


Fig. 8: Proposed fragmentation mechanism of DP_3 (m/z-404)

MS/MS degradation peak

The fragmentation mechanism of degradation product 3 of m/z-404 observed under the reduction degradation condition is shown in fig. 8. Abundant product ions are seen on the spectrum at m/z-343 (loss of $C_{10}H_9O_2$ from m/z-404), m/z-327 (loss of C_6H_5 from m/z-404), m/z-205 (loss of $C_6H_4NO_2$ from m/z-327), m/z-122 (loss of $C_4H_4O_2$ from m/z-205). The MS/MS experiments combined with mass measurements have confirmed the proposed structures.

MS/MS degradation peak

Fig. 9 shows the proposed fragmentation mechanism of degradation product 4 of m/z-492, observed under conditions of acid and alkali degradation. Abundant substance ions are seen on the spectrum at m/z-415 (loss of C_6H_5 from m/z-492), m/z-328 (loss of $C_4H_7O_2$ from m/z-415), m/z-153 (loss of $C_3H_3NOCl_3$ from m/z-328). The MS/MS experiments, combined with accurate mass measurements, have confirmed the proposed structures.

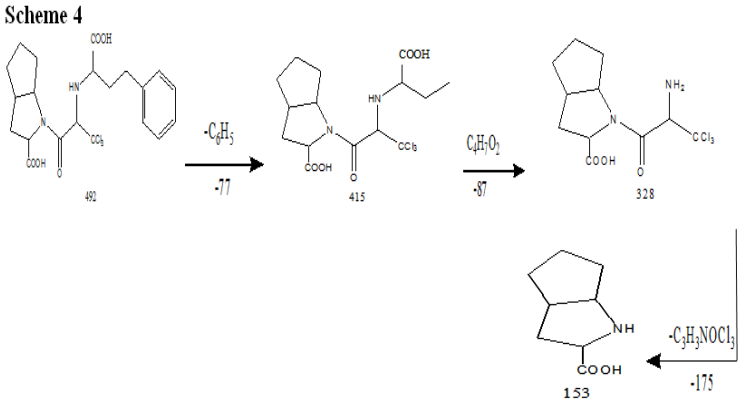


Fig. 9: Mechanism for proposed fragmentation of DP_4 (m/z-492)

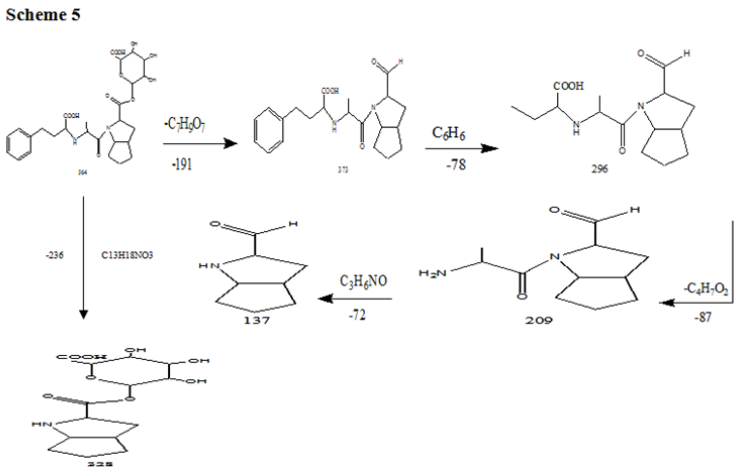


Fig. 10: Proposed fragmentation mechanism of DP_5 (m/z-564)

MS/MS degradation peak

The proposed fragmentation mechanism of degradation product 5 of m/z-564 observed under the condition of peroxide degradation is shown in fig. 10. Abundant productions are seen on the spectrum at m/z-373 (loss of $C_7H_9O_7$ from m/z-564), m/z-296 (loss of C_6H_5 from m/z-373), m/z-209 (loss of $C_4H_7O_2$ from m/z-296), m/z-137 (loss of C_3H_6NO from m/z-209), m/z-328 (loss of $C_{13}H_{18}NO_3$ from m/z-564). The proposed structures were confirmed by the MS/MS tests in combination with accurate mass measurements.

Scheme 6

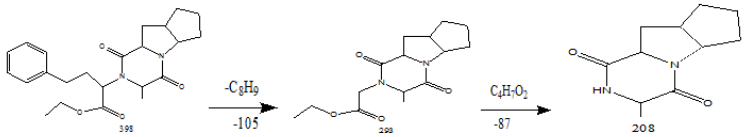


Fig. 11: Proposed fragmentation mechanism of DP_6 (m/z-398)

MS/MS degradation peak

The proposed fragmentation mechanism for degradation product 6 of m/z-398 observed under the reduction degradation condition is shown in fig. 11. The spectrum shows abundant product ions at m/z-293 (loss of C_8H_9 from m/z-398), m/z-208 (loss of $C_4H_7O_2$ from m/z-293). The proposed structures were verified in combination with accurate mass measurements by the MS/MS experiments.

CONCLUSION

In this a novel, simple, fast, economical, sensitive and easily available method of HPLC was developed for the simultaneous determination of Cilnidipine and Ramipril in the form of tablet dosage. The main advantages of this method were there is no HPLC methods were reported. In this method, shorter run time, low price, accessibility, sensitivity, reliability and reproducibility these properties were important when a large number of samples are to be analyzed. The validation of all the parameters like linearity, accuracy, specificity, robustness, stability was done and found to be within the acceptable limit. For all the parameters, RSD were found to be less than 2 percent which the validity of the process is suggested and the results obtained by this process are in good agreement. So the proposed procedure was easily applied for routine analysis and pharmaceutical formulations of Cilnidipine and Ramipril in quality control laboratories without any preliminary separation.

ACKNOWLEDGEMENT

The author was thankful to the management of P B Siddhartha college of Arts and Science for their encouragement and Shree Icon Pharmaceutical Laboratories for providing laboratory equipment.

FUNDING

Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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