

A High Sensitive Quantification Method for Lercanidipine and Enalapril in Bulk and Pharmaceutical Formulations Using UHPLC/ESI-Q-TOF-MS

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Abstract: A new approach using ultra-high performance liquid chromatography-electrospray ionization-tandem mass spectrometric method (UHPLC/ESI-Q-TOF-MS) for the rapid qualitative and quantitative analyses of Lercanidipine and Enalapril in tablet formulation has been developed and validated. The chromatographic separation was achieved on Agilent, Zorbax Eclipse plus-C18, a column with 1.8 μm particles packing which enabled the higher peak capacity, greater resolution, increased sensitivity and higher speed of analysis. The gradient mobile phase, was consisting of Milli-Q water with 0.1% formic acid, and 90% acetonitrile in water with 0.1% formic acid (90:10, v/v), at a flow rate of 0.2 mL min⁻¹. In the present study, UHPLC/ESI-Q-TOF-MS was operated under SIM scan mode using electro-spray ionization (ESI) technique with positive ion polarity to profile the abundances of Lercanidipine and Enalapril, Indapamide was used as the internal standard. The major monoisotopic ion in the positive mode for Lercanidipine, Enalapril and Indapamide (Internal standard) were at m/z 377.42, 613.02 and 366.06 at retention time of 3.63 min, 3.02 min and 2.58 min respectively. The linear dynamic range was established over the concentration range of 1.0-20.0 ng mL⁻¹ (r^2 ; =0.999) for Lercanidipine and 2.0-15.0 ng mL⁻¹ (r^2 ; =0.997) for Enalapril. Furthermore, the intra-assay and inter-assay accuracy in terms of % RSD was in between -0.1–3.7 % in both Lercanidipine and Enalapril. The advantages of hybrid Q-TOF mass spectrometry is not only include quality detection capability, sensitivity, but also include accurate measurement, reliable chemical fragmentation, which makes the structure elucidations easier.

Key words: Lercanidipine • Enalapril • Chemical Fragmentation • ESI-Q-TOF-MS

INTRODUCTION

Obesity-related hypertension represents a condition frequently observed in current clinical practice characterized by a complex pathophysiological background and a very high cardiovascular risk profile, particularly in severely obese individuals. Among the pharmacological interventions, a combination of two antihypertensive drugs based on a calcium channel blocker and an angiotensin-converting enzyme inhibitor, with specific focus on lercanidipine/enalapril represents the most common recommended strategy aimed at achieving blood pressure control on 'obesity-related

hypertension' [1]. Lifestyle changes and dietary factors can improve blood pressure control and decrease the risk of associated health complications, although drug treatment may prove necessary in patients for whom lifestyle changes prove ineffective or insufficient [2, 3].

In recent years, Lercanidipine plus enalapril maleate fixed dose combinations is used in patients with hypertension who have not reached blood pressure targets after treatment with either lercanidipine or enalapril monotherapy [4]. Therefore, we developed a new method to precisely estimate the film coated tablets of lercanidipine/enalapril combinations.

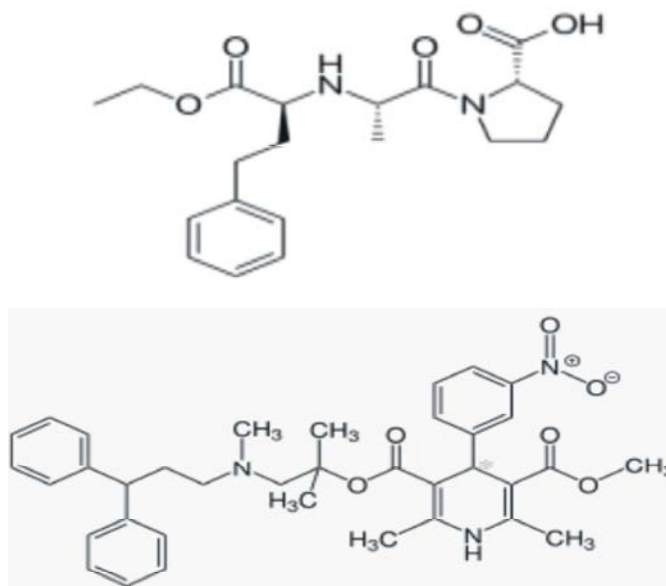


Fig. 1: Chemical structure of Enalapril and Lercanidipine

Then it was needed to confirm the bioequivalence of the developed formulations to the existing formulations. The analytical techniques particularly UV spectroscopy and other analytical techniques are important tools in assessing the quality of the products. Various analytical methods have been reported for the assay of Lercanidipine plus enalapril maleate in pure form as well as in pharmaceutical formulations. They include spectrophotometric estimation, RP-HPLC [5,6], GC-MS liquid chromatography with tandem mass spectroscopy [7]. Enalapril IUPAC name is (2S)-1-[(2S)-2-[[[(2S)-1-ethoxy-1-oxo-4-phenylbutan-2-yl]amino]propanoyl]pyrrolidine-2-carboxylic acid with molecular formula is $C_{20}H_{28}N_2O_5$ with average molecular weight 376.447 g/mol. Lercanidipine IUPAC name is methyl-1,1-dimethyl-2-(N-(3,3-diphenylpropyl)-N-methylamino)ethyl-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylates with molecular formula is $C_{36}H_{41}N_3O_6$ with average molecular weight 611.727 g/mol. Chemical structure of Lercanidipine and Enalapril is given in Fig. 1.

Till now, there is no ESI-Q-TOF method is reported for determining Lercanidipine/enalapril maleate in tablet dosage form. The objective of our work is to develop and validate a method for the determination of Lercanidipine/enalapril using ESI-Q-TOF. The LC-MS method has high specificity and sensitivity, since the limit of quantification of Indapamide was found ng/mL in reports [8-15]. In this study, we used ESI-Q-TOF analyzer to obtain high sample throughput. We have developed a rapid, simple, specific, and sensitive method for the determination of Lercanidipine/enalapril employing

coupled to high-performance liquid chromatography with tandem mass spectrometry detection (LC-MS/MS). We also report the validation for the method and application to a quantification of formulations of Lercanidipine/enalapril tablets.

MATERIALS AND METHODS

LC grade acetonitrile was purchased from J.T. Baker. Deionized (18 MX N cm) water was generated using a Milli-Q System (Millipore, Bedford, MA, USA) which was used for mobile phases as well as sample preparation. LC grade methanol was purchased from Fluka. All other chemicals and solvents were of analytical grade. Lercanidipine and enalapril tablets were procured from local pharmacy.

Instrumentation: The UHPLC-MS system consisted of an UHPLC system (Agilent 1290, Agilent Technologies) with diode array detector (DAD) coupled to quadrupole (Q-TOF) mass spectrometer (Agilent Technologies, USA) equipped with an ESI probe. MassHunter Quantitative Analysis (version 5.0) was used for data acquisition and processing.

Chromatographic Conditions: Chromatographic separation with UV detection was performed on a silica-based Zorbax Eclipse plus-C18 column (Rapid Resolution HD, 2.1 x 50 mm, 1.8 μ m particle size, Agilent, USA) at 25°C and with a gradient of two mobile phases. The flow rate was 0.2 mL/min and the injection volume

Table 1: Gradient program

Gradient programme time (min.)	Solvent B (%)
0	2
0.5	40
4.0	90
4.5	90
4.8	2
5.5	2

Table 2: Linearity data of Lercanidipine and Enalapril

Theoretical conc. (ng/ μ L)	Lercanidipine			Theoretical conc. (ng/ μ L)	Enalapril		
	Observed conc. in ng/mL (n=3) ^a	Precision (%) ^b	Accuracy (%) ^c		Observed conc. in ng/mL (n=3) ^a	Precision (%) ^b	Accuracy (%) ^c
1.0	1.1 \pm 0.1	7.2	112.7	2.0	1.7 \pm 0.1	6.2	86.6
5.0	4.8 \pm 0.1	1.5	96.1	5.0	5.4 \pm 0.2	4.0	107.9
10.0	10.0 \pm 0.1	1.3	99.5	7.0	6.9 \pm 0.5	6.9	98.4
15.0	15.2 \pm 0.2	1.5	101.3	10.0	10.1 \pm 0.5	5.1	100.9
20.0	19.9 \pm 0.2	1.2	99.6	15.0	14.9 \pm 0.5	3.3	99.3
Intercept	6898.3			Intercept	954.5		
Slope	20976.9			Slope	2132.6		
Correlation	0.999			Correlation	0.997		

a) Mean \pm SD.

b) RSD, $100 \times (\text{SD}/\text{mean})$.

c) $[(\text{observed conc.} - \text{theoretical conc.})/\text{theoretical conc.}] \times 100$

was 10 μ L. The vial temperature was maintained at 5-8°C in the auto sampler tray. Mobile phase solvent A consisted of Milli-Q water with 0.1% formic acid, and mobile phase solvent B consisted of 90% acetonitrile in water with 0.1% formic acid (90:10, v/v). The gradient elution was programmed for total 5.5 min, which consist of increasing solvent B from 0 % to 2 % over 2 min, and increased to 90 % over 4.0 min and then conditioning the column back to initial conditions. The complete gradient conditions are mentioned in Table 1.

Mass Spectrometric Conditions: For on-line UHPLC MS experiments the chromatographic conditions were maintained the same as for the method with UV absorbance detection. Column effluent was diverted to waste from 0 min to 0.5 min, and from 4.5 min to 5.5 min next run, in order to avoid ion source contamination. UHPLC effluent was sprayed directly into the mass spectrometer at a flow rate of 0.2mL/min. The Q-TOF mass spectrometer was operated with the ESI probe in positive mode at open resolution. Lercanidipine and enalapril was detected in scan mode using the following software settings: mass range 100-800m/z, Acquisition rate/time used 2 spectra/seconds. Ion source and other instrument parameters were optimized for scanning and the following settings were used: Fragmentor 175V, Skimmer 65V, OCTIRF Vpp 750V, Gas temperature 300°C, drying gas 8L/min and nebulizer 45 psig. In order to confirm the

analyte, structural characterization study was performed using in MS/MS mode using 20V collision energy by keeping the above said method source conditions. The described conditions were optimized to achieve best sensitivity for analyte detection. The fragmentation of Lercanidipine and enalapril (Figs. 2 and 3) shows the structural confirmation.

Preparation of Standard Solutions and Test Samples:

A pure drug of about 10 mg Lercanidipine and enalapril powder weighed separately and transferred in to 10 ml volumetric flasks and dissolved in 5 ml of 50 % methanol by shaking and volume was made up to the mark with 50 % methanol to obtain final concentration of 1 mg/ml of stock solution. The stock solution was further diluted to 10 μ g/mL and used for tuning of Lercanidipine and enalapril analysis. From this 10 μ g/mL stock solution, various standard solutions of Lercanidipine and enalapril was prepared by 70% methanol in MilliQ water with 0.1% formic acid to obtain the concentrations of 1.0, 5.0, 10.0, 15.0 and 20.0 ng/mL for Lercanidipine. The same way enalapril was prepared to obtained the concentrations of 2.0, 5.0, 7.0, 10.0 and 15.0 ng/mL.

To prepare the test sample for the estimation of Lercanidipine and enalapril in tablet formulation, 20 tablets were weighed and triturated to fine powder. Tablet powder equivalent to 10mg of Lercanidipine and 20 mg of enalapril was weighed, dissolved and further diluted with

sufficient quantity of 50 % methanol. It was sonicated for 20 min and then filtered through Whatmann filter paper no. 41 to get the stock solution of 1000 µg/ml. Various dilutions of the tablet solution were prepared and analyzed for three times and the concentration was calculated by using the calibration curve method (Table 2).

RESULTS

Method Development: The peak at an approximate retention time of 3.63 min and 3.02 min was selectively detected after separation on Zorbax eclipse plus C18 column by positive ion mode ESI-MS at 613.02–280.35 (Fig. 2) for Lercanidipine at the retention time of 3.02 min and 377.42–234.35 for enalapril at the retention time of 3.63 min (Fig. 3). For internal standard Indapamide was separated at the retention time of 2.58 min with 366.06–132.08 (Fig. 4).

By varying the ionization conditions in the mass spectrometer and after chromatographic elution a well separated and identified peak was observed, it was clear that the observed Lercanidipine, enalapril and Indapamide were produced as a direct consequence of the ESI process in the ion source. The most intense signal transition in fragmentation with m/z 280 for Lercanidipine (Figure 2), with m/z 234.35 for enalapril and with m/z 132.08 for internal standard Indapamide (Figure 3) were selected for quantification in MRM mode (Fig. 4). The ESI-MS/MS method was originally developed and optimized for a gradient UHPLC method with 5.5 min run time performed on an Zorbax eclipse plus C18 column, which confirm the presence of Lercanidipine, enalapril and Indapamide structures.

Specificity: Specificity was assessed by injecting Lercanidipine, enalapril and Indapamide samples dissolved in 50 % methanol and blank solution without spiking of Lercanidipine, enalapril and Indapamide. No co-elution with Lercanidipine, enalapril and Indapamide at retention time 3.5 min, 3.02 min and 2.58 min respectively, or any other component of significant response at the given transition was observed (Fig. 5).

Separation performed on zorbax eclipse plus C18 column sufficiently retained Lercanidipine, enalapril and Indapamide while separating the analyte of active ingredient from other mixtures of excipients in formulated tablets (e.g. matrix forming agent, diluent, lubricant, antiadherent and flow promotor). Special care (Film coated tablet care) was taken to confirm that Whatmann filter

paper membrane did not interfere with the analysis by polluting the flow-through solution with membrane interferences.

Linearity: The standard linearity was evaluated by triplicate injections of standard solution following the same procedure for standard and test samples. The concentration ranges were the same as shown in Table 2. Sample linearity data were obtained from the accuracy study and the calculated correlation coefficients obtained through MS detection was presented in Table 2. A five point calibration curve was used for the quantification of Lercanidipine and enalapril in MS detection. In both Lercanidipine and enalapril, detector responses indicated a good correlation between concentration and peak area with acceptable deviations and estimated linearity parameters. The regression curve was constructed by linear regression fitting and its mathematical expression was $Y = mX + c$ (Where Y gives measured concentration and X is the theoretical concentration of the Lercanidipine and enalapril, m is slope and c is intercept, determines the point at which the line crosses the y-axis) and the coefficient of determination (R^2) was found to be ≥ 0.999 and ≥ 0.997 respectively.

The estimated LOQ for Lercanidipine and enalapril in test samples was 1.5 ng/µL and 2.2 ng/µL respectively, considering accuracy and precision acceptance limit within 10% for both Lercanidipine and enalapril. The obtained LOD for Lercanidipine and enalapril was 0.2 ng/µL and 0.5 ng/µL respectively, based on a signal-to-noise ratio higher than 3. However, LOD for MS detection can vary from 0.1-1.5 ng/µL relative to the ion source conditions (e. g. source contamination) for both Lercanidipine and enalapril. Both the LOQ and LOD were determined by serial dilutions of test samples.

Accuracy and Precision: Intra-day accuracy expressed as $[(\text{observed conc.} - \text{theoretical conc.})/\text{theoretical conc.}] \times 100$ and precision ($\text{SD}/\text{mean conc.} \times 100$, $n = 6$ for each level) were evaluated by analysis of three different concentration of test samples on the same day. Inter-day accuracy and precision were determined by repeated analysis over three consecutive days. Accuracy and precision were evaluated by analyzing test sample solutions prepared in the concentration level 2.5-12.5 ng/mL for both Lercanidipine and enalapril and 50 ng/mL fixed concentration of Indapamide was used as internal standard throughout the analysis. Detection was achieved in higher sensitivity by scan mode. Results for accuracy and precision in scan mode

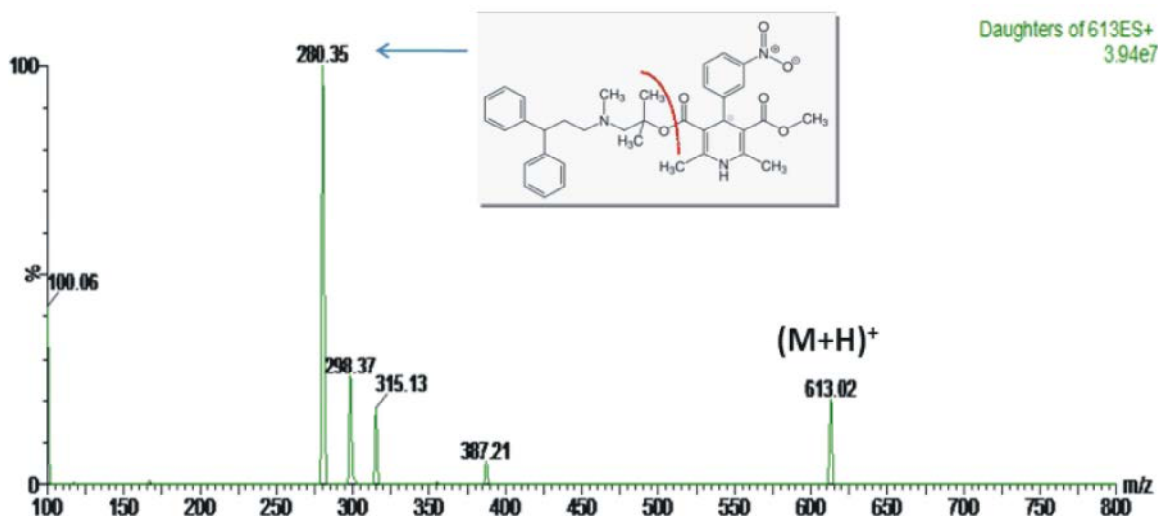


Fig. 2: A well separated Lercanidipine (10 $\mu\text{g/mL}$) in TIC chromatogram with transition at m/z 613.02_280.35 obtained at 20 eV collision energy, which confirm the presence of Lercanidipine structure

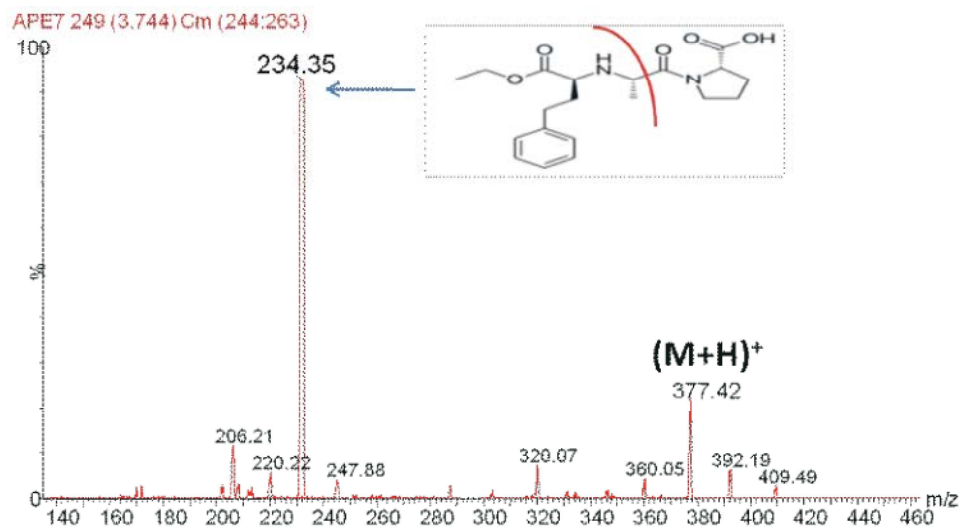


Fig. 3: A well separated Enalapril (10 $\mu\text{g/mL}$) in TIC chromatogram with transition at m/z 377.42_234.35 obtained at 20 eV collision energy, which confirm the presence of Enalapril structure

Table 3: Intraday Accuracy and precision data of Lercanidipine and Enalapril

Intra-day variation							
Lercanidipine				Enalapril			
Observed conc.				Observed conc.			
Theoretical conc. (ng/ μL)	in ng/mL (n=3) ^a	Precision (%) ^b	Accuracy (%) ^c	Theoretical conc. (ng/ μL)	in ng/mL (n=3) ^a	Precision (%) ^b	Accuracy (%) ^c
2.5	2.6 \pm 0.1	3.7	105.5	2.5	2.3 \pm 0.1	3.0	91.5
7.5	7.3 \pm 0.4	5.7	96.9	7.0	7.3 \pm 0.2	3.2	104.3
12.5	12.0 \pm 0.3	2.6	97.1	12.5	12.3 \pm 0.5	3.8	98.2

Table 4: Inter-day Accuracy and precision of Lercanidipine and Enalapril

Lercanidipine				Enalapril			
Observed conc.				Observed conc.			
Theoretical conc. (ng/ μ L)	in ng/mL (n=3) ^a	Precision (%) ^b	Accuracy (%) ^c	Theoretical conc. (ng/ μ L)	in ng/mL (n=3) ^a	Precision (%) ^b	Accuracy (%) ^c
2.5	2.4 \pm 0.1	2.2	96.5	2.5	2.5 \pm 0.1	2.1	99.0
7.5	7.4 \pm 0.2	2.1	98.3	7.0	7.2 \pm 0.3	3.9	103.5
12.5	12.4 \pm 0.3	2.3	99.3	12.5	12.1 \pm 0.4	3.7	96.8

a) Mean \pm SD.

b) RSD, 100 \times (SD/mean).

c) [(observed conc.-theoretical conc.)/theoretical conc.] \times 100

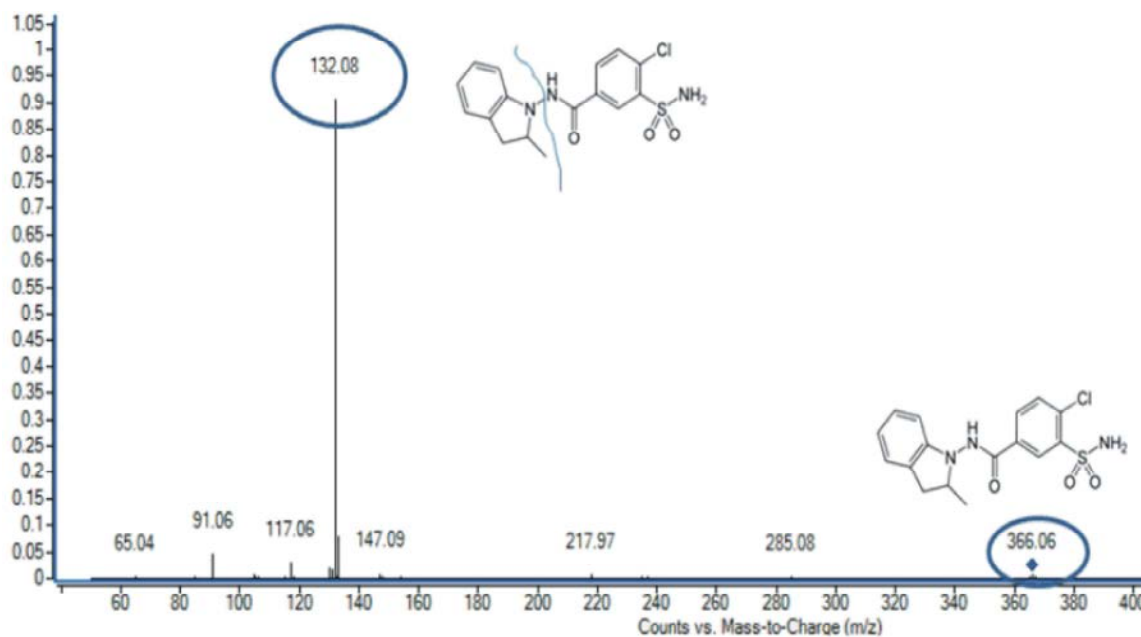


Fig. 4: Positive ion mode ESI-MS/ MS spectrum of Indapamide (10 μ g/mL) at m/z 366.06–132.08 obtained at 20 eV collision energy, which confirm the presence of Indapamide structure

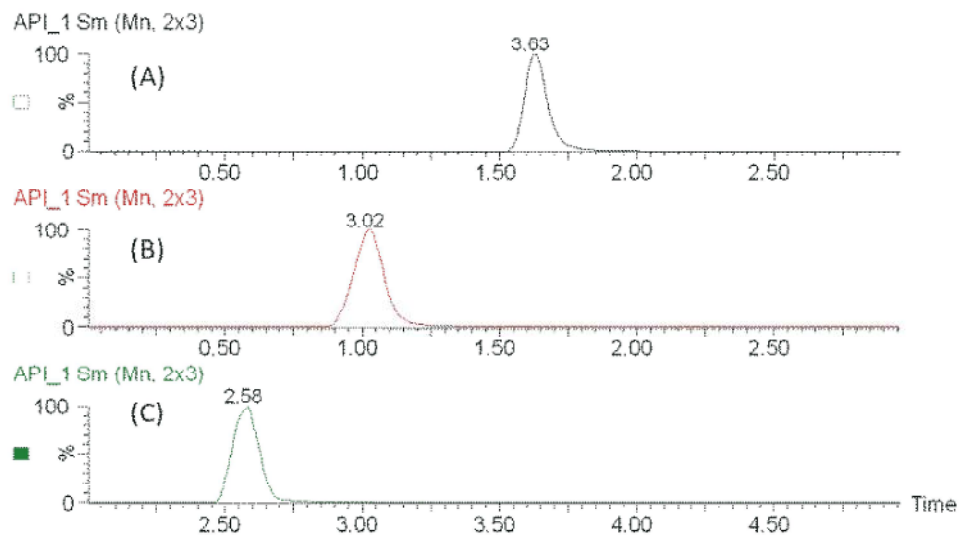


Fig. 5: A well separated TIC of Lercanidipine (A), Enalapril (B) and Indapamide (C) at 10 μ g/mL

are presented in Tables 3 and 4. The intraday accuracy calculated in the range of 96.9- 105.5 % for Lercanidipine, and 91.5-104.3 % for enalapril while the precision was in the range of 2.6-5.7 % for Lercanidipine, and 3.0-3.8 % for enalapril (Table 3). The inter-day accuracy was in the range of 96.5-99.3 % for Lercanidipine, and 99.0-103.5 % for enalapril, the precision (% RSD) was not more than 2.3 % for Lercanidipine, and not more than 3.9 % for enalapril (Table 4).

During the assay development carryover was not observed at any rate, even after consecutive injections at the highest concentration level. The stability of the analyte tested within 24 h by MS and MS/MS method and it did not affect any evaluated method parameter.

Recovery: For determining the % recovery, the data obtained from linearity study was considered and the % recoveries were calculated. These are compared with the theoretical values to obtain the % recovery. Accuracy was determined by assaying 3 times by the same analyst, on three different days. The % recovery was calculated with respect to the nominal concentration by using below equation, % Recovery = (Observed concentration/Theoretical concentration) \times 100. The % recovery was between 96.1 and 112.7 % with a precision lower than 7.2%, expressed as % RSD for Lercanidipine and for enalapril 86.6 and 107.9 % with a precision lower than 6.9 %, expressed as % RSD (Table 2).

DISCUSSION

In order to achieve optimum separation of the component Lercanidipine and enalapril peak, mixtures of 0.1% formic acid and 90 % acetonitrile in different combinations were used as mobile phase on a C18 stationary phase. The retention time obtained for Lercanidipine and enalapril was 3.63 and 3.02 min respectively.

When Lercanidipine and enalapril solutions were analyzed by the proposed method for finding out intra and inter-day variation, $\leq 10\%$ co-efficient of variation was observed. There was no interfering peaks, which indicates the selectivity of the method. Recovery values within $100 \pm 20\%$ obtained from the three different levels spiking by the proposed method indicates the method was accurate.

The value of LOD and LOQ obtained by the proposed method indicates the sensitivity of the method. Hence it can be concluded that the proposed LC-MS method is sensitive and reproducible for the analysis of Lercanidipine and enalapril in tablet formulation with short

analysis time of 5.5 min. The evaluation of results obtained in this study demonstrates that the method for estimation of Lercanidipine and enalapril content in tablet formulation samples satisfies the necessary requirements for qualification. Based on this qualification data we can conclude that this LC-MS technique is sensitive, precise and specific to identify and quantify Lercanidipine and enalapril in formulated tablet samples.

CONCLUSIONS

A direct and simple UHPLC method using MS detection was developed for Lercanidipine and enalapril quantitation in tablet formulation. The advantages of fully end capped stationary phases were utilized to retain Lercanidipine and enalapril on the column, while separating the analyte of active ingredient from other mixtures of excipients in formulated tablets. Time-consuming reduction, derivatization, and HPLC fluorescence detection can be readily replaced by reversed-phase UHPLC separation coupled with ESI-MS detection in positive ion mode, which provides reasonable results for precision, accuracy, and linearity in approximately the same evaluated concentration ranges. In addition, a less sensitive chromatographic method based on the same principles can be implemented for quantitative analysis of more concentrated Lercanidipine and enalapril samples using only MS detection (approximately 2.0 ng/ μ L or higher).

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CONFLICT OF INTEREST: The authors declared that there is no conflict of interest.

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