Development and validation of a sensitive LCMS/MS method for the simultaneous quantification of Azelnidipine and Chlorthalidone in human plasma

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Abstract
Highly resolved, selective and sensitive liquid chromatography/tandem mass spectrometry (LC–MS/MS) method was optimised for the simultaneous quantification and recently approved combined medication for the treatment of hypertension viz. azelnidipine and chlorthalidone in spiked human plasma. The study utilizes nifedipine as internal standard. In the extraction of analytes from spiked plasma, acetonitrile was used to induce protein precipitation and then liquid – liquid extraction was carried using ethyl acetate solvent followed by the LCMS-MS analysis. The analytes were separated on Inertsil ODS C18 (4.6 mm×100 mm, 5 µm) column at room temperature with 65:35 (v/v) ratio of acetonitrile and 0.1% formic acid as the mobile phase at 0.5 mL/min flow rate. Detection was performed on a triple quadrupole mass spectrometer employing electrospray ionization technique, operating in multiple reaction monitoring with the transitions of m/z 583 → 331, 339 → 321 and 347 → 328 respectively for azelnidipine, chlorthalidone and nifedipine.

The analytes were detected at a retention time of 1.72 min, 2.62 min and 3.12 min respectively for azelnidipine, chlorthalidone and nifedipine and the analysis was completed within run time of 5 min. The analysis was carried in the calibration range of 5-500 ng/mL with a very sensitive detection limit of 1.5 ng/mL. The method was validated as per FDA guidelines for bioanalytical method validation and the results in all the validation parameters were in the acceptable limit. Results proved that the method was selective, sensitive, accurate, precise and stable and hence can be applicable for the routine analysis of azelnidipine and chlorthalidone in biological samples.

Keywords: Azelnidipine, Chlorthalidone, Bio-analytical method, LCMS/MS analysis, Spiked human plasma.

Introduction

Azelnidipine (Figure 1A) is an antagonist of dihydropyridine calcium channel and is a selective L-type calcium channel antagonist approved for the treatment of adult patients suffering with hypertension[4]. The antihypertensive effects of azelnidipine were comparable with amlodipine[5]. In comparison with the other old generation calcium channel blockers, azelnidipine was lipid soluble and shows high vascular wall selectivity and significantly increases the blood flow to the brain[8].

It was available in the form of tablets administered orally. It adsorbs rapidly and shows dose-dependent absorption. Slow heart rate and edema are the most common side effects and jaundice, headache, skin rash, redness of skin and dizziness are rare side effects associated with the use of azelnidipine[3]. Its molecular formula is $C_{33}H_{34}N_4O_6$ and molecular mass of 582.657 g/mol.

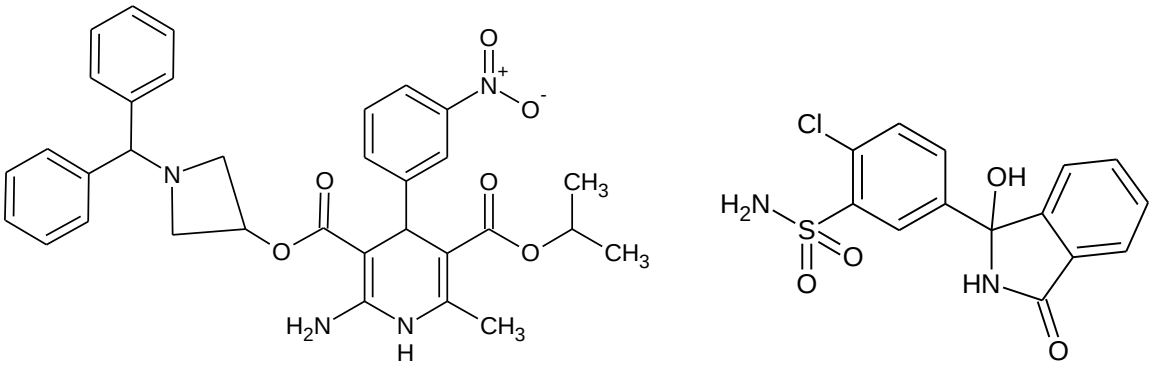
Chlorthalidone (Figure 2A) belongs to diuretics class thiazide-like drug prescribed for the treatment of high blood pressure, liver failure, diabetes insipidus, nephrotic syndrome, swelling including heart failure and renal tubular acidosis[1]. It was suggested as initial treatment for the patients with high blood pressure. It was also prescribed to prevent kidney stones formed due to calcium[1]. In preventing the heart stroke and heart attacks, it works more effective than hydrochlorothiazide[6].

The common side effects possible while using chlorthalidone include low blood sodium, dizziness, low blood potassium, erectile dysfunction and high blood sugar whereas the high blood calcium, low blood magnesium, gout and allergic reactions are rare side effects[2].

In the treatment of essential hypertension, the fixed dose combined dosage form of azelnidipine and chlorthalidone was approved by Drugs Controller General of India in the year 2021. Methods available for the estimation of azelnidipine in single or in combination with other drugs and chlorthalidone in single or in combination with other drugs are various analytical techniques such as HPLC, LCMS, UPLC etc. In view of this, the present study was focused in the development and validation of simple LCMS MS method for the simultaneous estimation of azelnidipine and chlorthalidone in human plasma. Nifedipine which is also a calcium channel blocker having similar therapeutic activity of the analytes in the study, was selected as internal standard and its molecular structure is shown in figure 2.

Material and Methods

Materials: The standard analytical pure drugs in the study viz. azelnidipine and chlorthalidone were procured from IPCA Laboratories Ltd., Hyderabad and the internal standard drug nifedipine was obtained from Cipla Limited, Hyderabad.



A) **B)**

Figure 1: Molecular structure of azelnidipine (A) and chlorthalidone (B)

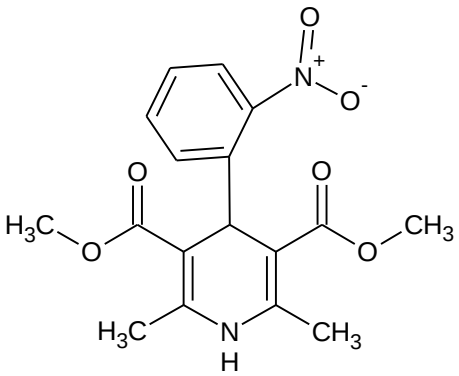


Figure 2: Molecular structure of nifedipine (internal standard)

The HPLC grade chemicals in the stuyd (methanol, acetonitrile) and milli Q water were purchased from Merck chemicals, Mumbai. Healthy human blood was procured from nearby diagnostic laboratory. The plasma from the whole blood was separated using Pasteur pipette after centrifugation.

Equipment: The experiment was performed on Waters (Japan) alliance 2695 LCMS system coupled with triple quadrupole mass detector (Waters ZQ, LAA 1369). The system was equipped with auto-injector (0. 1 –1500 µL) and integration was carried on masslynx 4.2 (waters) software. The analytes was separated on Inertsil ODS C18 (4.6 mm×100 mm, 5 µm) column.

Preparation of working standard solutions: The standard stock solution of azelnidipine and chlorthalidone was prepared separately in 25 mL volumetric flask by accurately weighing and dissolving 25 mg of analyte in 15 mL of methanol. Then the flask was sonicated for 2 min to dissolve the analytes in solvent and the final volume was made up to the mark using the same diluent.

The standard azelnidipine and chlorthalidone at a concentration of 1000 µg/mL were obtained separately. Then the stock solutions were diluted successively to obtain a standard solution in the concentration range of 5 to 500 ng/mL separately for azelnidipine and chlorthalidone. The same procedure was followed for the preparation of nifedipine internal standard solution at a concentration of 200 ng/mL separately.

Preparation of spiked calibration curve standard solutions: The calibration curve dilutions were prepared by spiking 50 µL of prepared standard concentrations of azelnidipine and chlorthalidone in increasing order of 50 µL of internal standard to the blank human plasma. Similarly, a blank without analytes and a zero sample that spiked with internal standard only were prepared. All the spiked plasma samples were treated as per the extraction protocol.

Extraction protocol: The protein precipitation of spiked plasma was achieved by adding acetonitrile (1 mL) and the extraction of analytes was performed using ethyl acetate (3 mL). The content was vortexed for 2 min and then centrifuged at 10 °C for 5 min at 400 rpm. The supernatant obtained was carefully relocated and concentrated at 60 °C and reconstituted with methanol. The reconstituted solution was used for the LCMS analysis.

Method development: The method development for the separation and analysis of azelnidipine and chlorthalidone in presence of nifedipine internal standard was carried by performing the analysis in different analytical conditions. One parameter was changed and other parameters were kept constant. The spiked standard solution was analysed in each changed conditions. The results achieved in each condition was observed and the conditions that give the best results in

terms of system suitability, specificity and symmetry were selected for further validation.

Method validation: The developed method for the analysis of azelnidipine and chlorthalidone using LCMS was validated as per the guidelines of FDA[7].

Selectivity: The method selectivity was evaluated by analysing and comparing the results achieved for the un-spiked (blank) plasma matrix and the analytes at LLOQ level spiked plasma matrix. The results achieved in theses analysis were compared and the selectivity of the method was assessed.

Linearity and range: The range of azelnidipine and chlorthalidone was assessed by analysing the plasma spiked calibration curve dilutions prepared in the developed method. Peak area response ratio of each analyte to the internal standard was plotted against the concentration of the analyte prepared. The linear regression analysis was performed to assess the linearity of the analytes in the developed method.

Accuracy and precision: The accuracy and precision were performed as intraday and interday studies and were carried in three dissimilar concentrations such as low (LQC), middle (MQC) and high (HQC) concentrations in the linearity range. The selected concentrations in the linearity range were analysed in six replicates for both intraday and interday precision studies. The results in the study were expressed as the % relative standard deviation and the % recovery for all the studied levels. The results concluded as acceptable when the variation results were within ± 15% in the three studied levels.

Recovery: The recovery of the method developed was evaluated by comparing the results observed during the analysis of analytes that were spiked with blank plasma which was exposed to the whole extraction procedure to the results observed for post-extracted plasma samples. The study was conducted at LQC, MQC and HQC levels in the calibration range. It can be considered that the recovery of the analytes may not be 100 % but it is essential that the variability of the results in recovery must be reproducible, precise and consistent in different concentration ranges.

Matrix effects: The matrix effect of the method developed for the analysis of azelnidipine and chlorthalidone was evaluated by investigating the effect of blank plasma on the results. In this, the blank plasma of six different batches was spiked with the investigated analytes at LQC and HQC levels and was analysed by the developed method. The %RSD of the peak area response of both the analytes in the study was calculated and % RSD of < 15 % confirmed that the method was having acceptable matrix effect.

Dilution integrity: The effect of dilution on the accuracy and precision of the analytes in the developed method was assessed in this study. The study was conducted by spiking the analytes at higher than the HQC concentration and then the sample was diluted to HQC and LQC level with the blank plasma matrix. The method was acceptable if the precision and accuracy were within ± 15%.

Stability experiments: The stability analysis was conducted to evaluate the stability of the analytes in plasma matrix under several conditions which simulate the conditions that could occur during sample analysis. Different stability studies such as short term, long term, auto sampler, freeze and thaw and dry extract stability were performed at LQC, MQC and HQC levels utilizing six replicates from each level.

In short term stability, the defrosted samples were stored at room temperature for 6 h and then analysed in the developed method. In long term stability, the samples were stored in freezing temperature and were analysed after 30 days of the incubation. In freeze thaw stability, the QC samples stability was investigated through four freeze–thaw cycles after being kept to freeze for 24 h. Samples were then thawed unassisted at room temperature for 2 h or even more and then kept to freeze at − 86 °C overnight for every freeze–thaw cycle.

The consequences of infrequent delay of the sample injection in auto-sampler were evaluated in auto-sampler stability. In this, the sample was analysed after 24 h of incubation in an auto-sampler. The dry extract stability was assessed by incubating the dry residue at room temperature without reconstitution and was reconstituted after 24 h of incubation. The % recoveries and the % stability in all the studies were calculated in the studied concentration levels for both the analytes.

Results and Discussion

To develop a highly sensitive and robust analytical method, the solid-phase extraction and the liquid–liquid extraction were widely used for the preparation of samples from the biological samples. The solid-phase extraction technique was treated as an expensive and it was very difficult to extract the highly polar compounds through liquid – liquid extraction. In view of this, the present study utilises a simple protein precipitation technique for the prepration of samples and it was done using acetonitrile solvent. It facilitates the higher efficiency for precipitations of proteins with very less analyte lose when compared with other solvents such as acetone and methanol. Further the protein precipitation was followed by liquid – liquid extraction using ethyl acetate solvent.

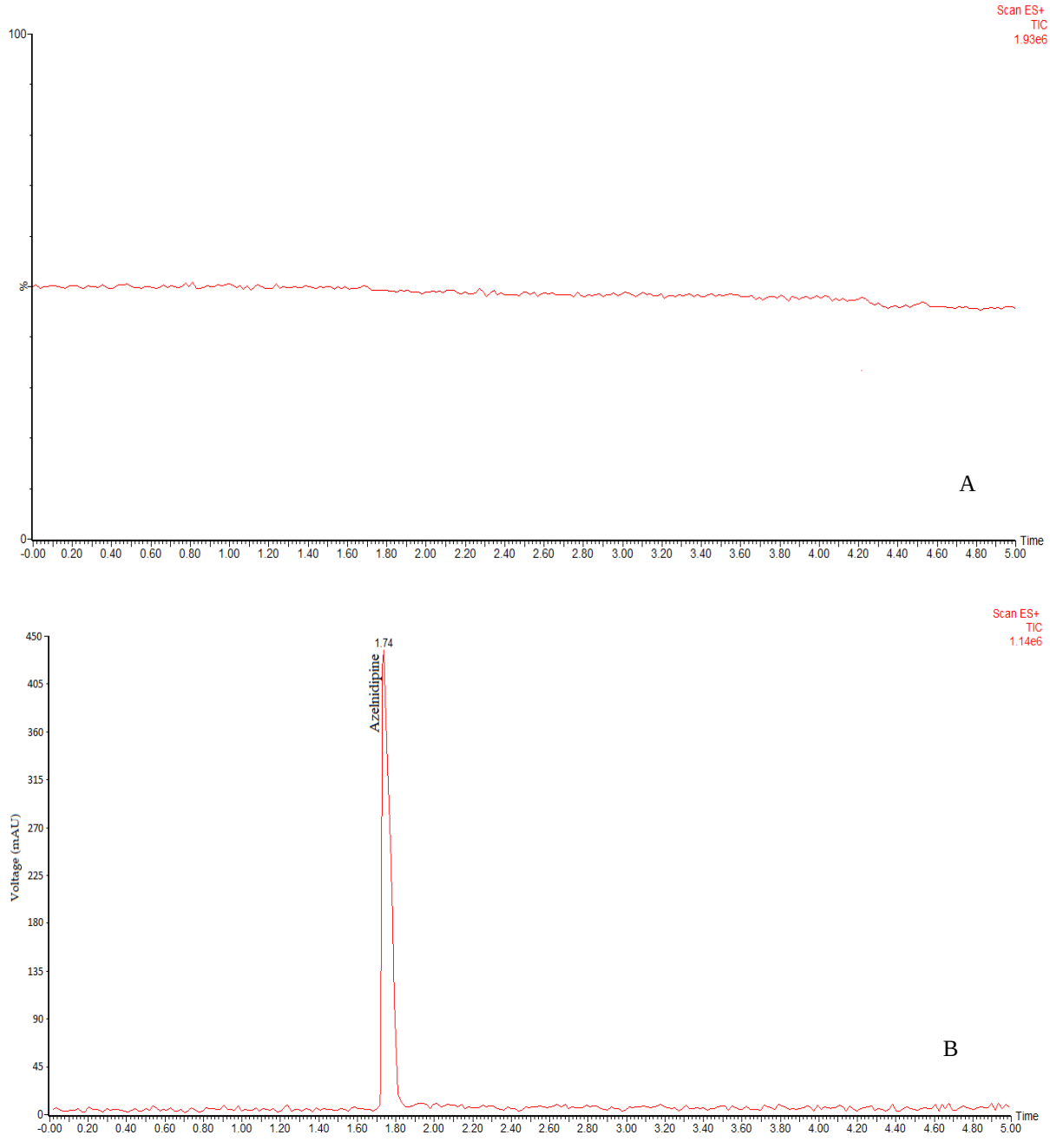
The extracted samples were analysed in the method development followed by method validation study using LCMS. The analytes in the study viz. azelnidipine and chlorthalidone have dissimilar physicochemical properties; consequently, considerable effort was required to adjust the chromatographic conditions in order to achieve sharp peaks shape and satisfactory response. Various conditions were

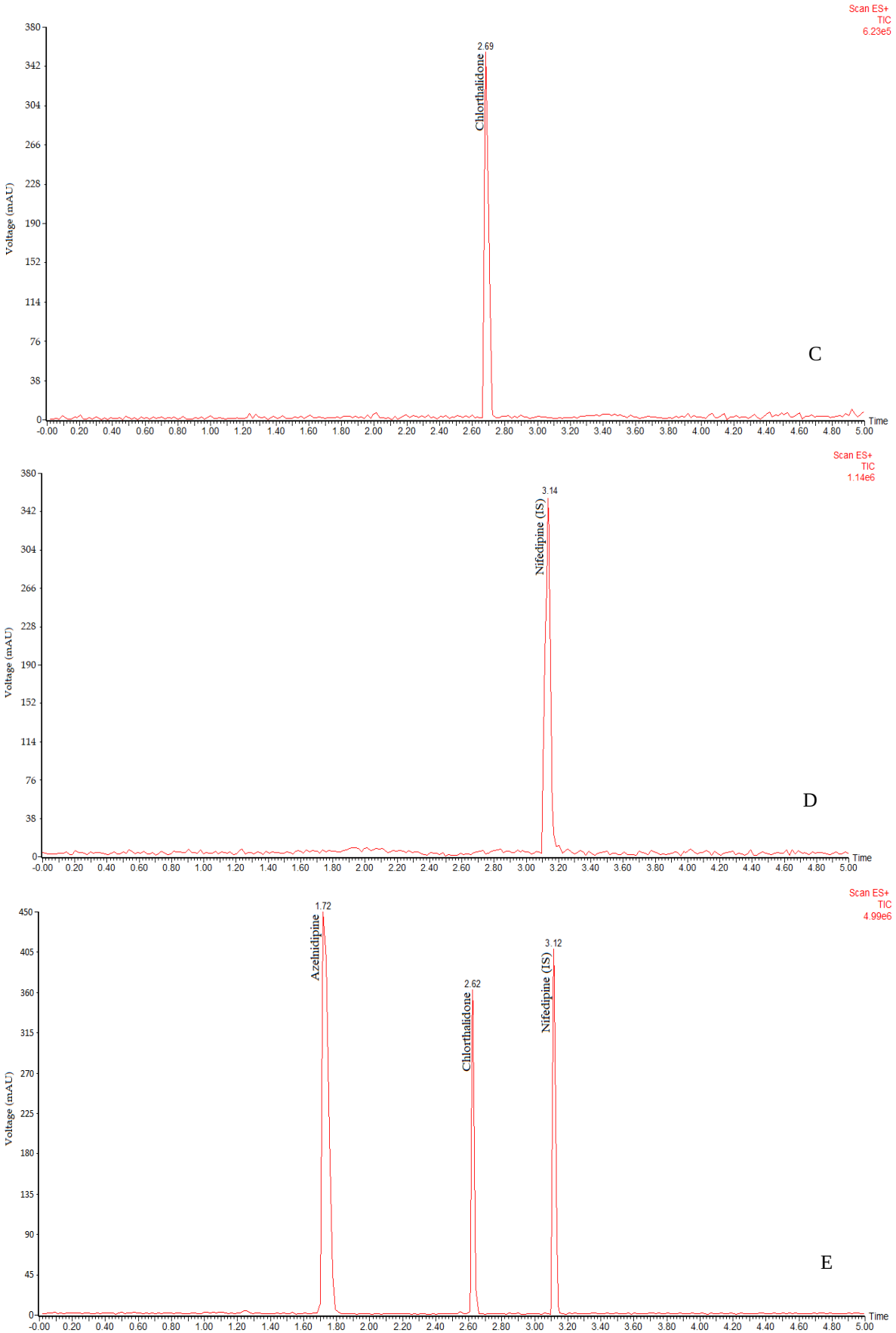
adjusted to attain suitable analytical conditions for the separation and simultaneous analysis of azelnidipine and chlorthalidone along with internal standard nifedipine. Very nominal quantity of sample (2 µL) was separated on Inertsil ODS C18 (4.6 mm×100 mm, 5 µm) column at room temperature with 0.5 mL/min flow rate of mobile phase that facilitates less consumption of mobile phase.

In the selection of mobile phase, acetonitrile and methanol at various compositions were studied as organic modifier and formic acid at various compositions and pH ranges. The involvement of acetonitrile as mobile phase produces best response than methanol and hence acetonitrile was used as organic modifier in the study. It was confirmed that 65:35 (v/v) ratio of acetonitrile and 0.1% formic acid as the mobile phase were most suitable for obtaining the best sensitivity, efficiency and peak shape.

The typical chromatograms observed in the developed conditions as shown in figure 3 confirm that the un-spiked chromatogram does not show any peak throughout the run time whereas the spiked standard chromatogram shows peaks at a retention time of 1.72 min, 2.62 min and 3.12 min respectively for azelnidipine, chlorthalidone and nifedipine.

The retention time observed for both the analytes in the combined standard solution was comparable with the individual spiked chromatogram. The results confirm that there is no interference of endogenous plasma components during the analysis and hence it proved that the method was specific for the analysis of analytes in the study.





A) un-spiked plasma sample; B) spiked with azelnidipine; C) spiked with chlorthalidone; D) spiked with internal standard (nifedipine); E) spiked with both analytes and internal standard

Figure 3: LCMS chromatograms obtained in the optimized conditions

Coupling of liquid chromatography to MS/MS detection is an extremely selective technique which results in insignificant interference of endogenous impurities. The mass detector operated in multiple reaction monitoring (MRM) mode was very influential method that produces sensitivity and the selectivity to the analytes. Hence in the study, the mass detector in MRM mode was selected for the detection of analytes. Both +ESI and −ESI were investigated and it was obvious that the signal intensities acquired from the +ESI were higher than those acquired from the −ESI which could be attributed to the capability of the target analytes as well as internal standard to gain protons.

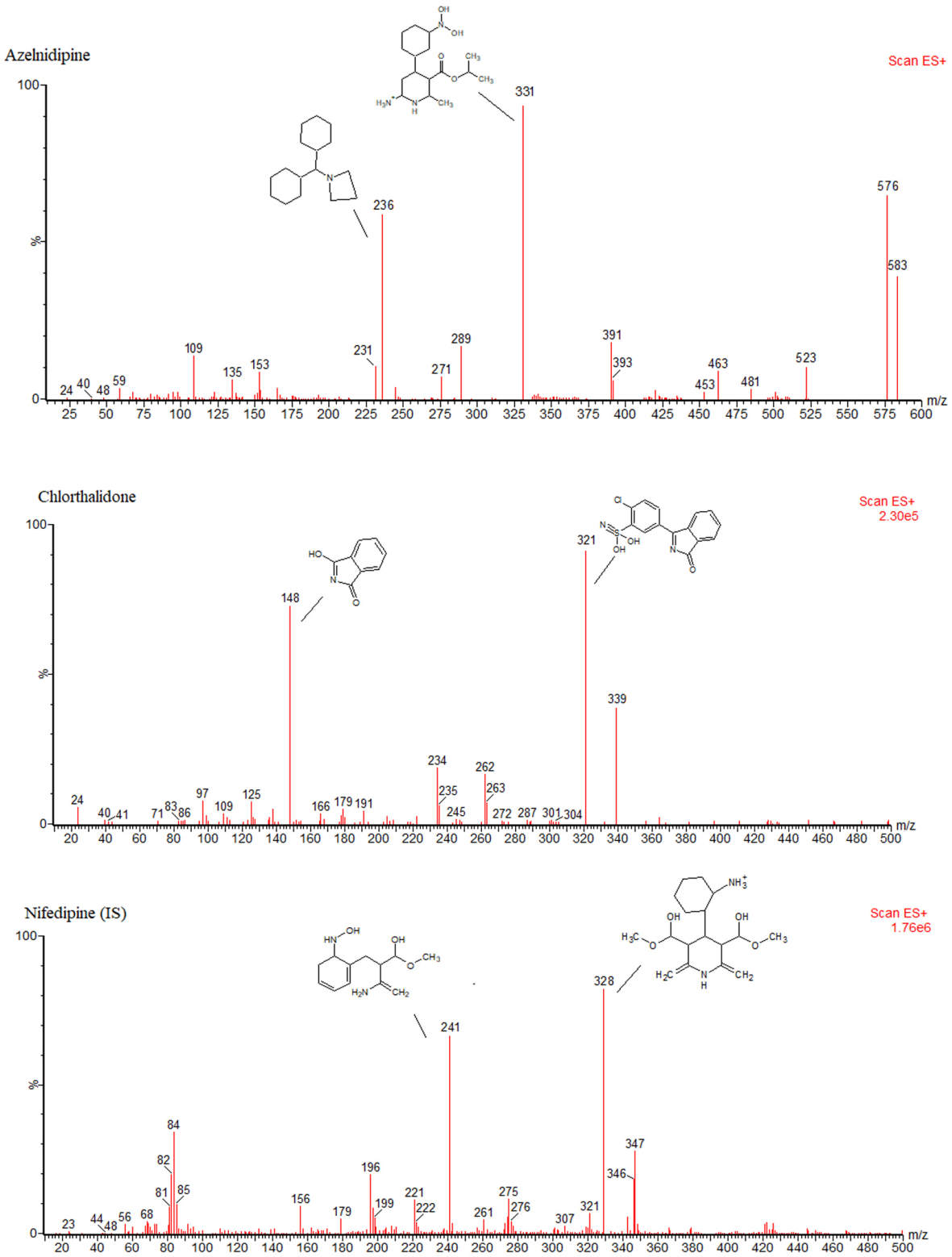


Figure 4: Full scan mass spectra observed in the optimised conditions

The full scan Q1 mass spectra show predominant protonated $[M+H]^+$ parent ions at m/z of 583, 339 and 347 for azelnidipine, chlorthalidone and nifedipine respectively. The characteristic most abundant fragment ions found in the product ion mass spectrum were 331, 321 and 328 respectively for azelnidipine, chlorthalidone and nifedipine. The mass spectral parameters were summarized in table 1 and full scan mass spectra observed for the analyst in the study are given in figure 4.

The calibration curve was observed to be linear in the concentration range of 5 to 500 ng/mL for both the analytes with regression equation of y = 0.0059x - 0.0017 (R^2 = 0.9992) and y = 0.0045x + 0.004 (R^2 = 0.9998) for azelnidipine and chlorthalidone respectively. The results of the linearity (table 2) confirm that the method has broad and sensitive calibration curve.

The precision and accuracy of the method developed for the analysis of azelnidipine and chlorthalidone using LCMS were evaluated in HQC, MQC and LQC levels. The % accuracy was observed to be in the range of 94.55 - 100.57 % with % RSD in the range of 1.11 - 1.40 % for azelnidipine whereas for chlorthalidone, the % recovery was obtained in the range of 94.23 - 101.98 % with the % RSD in the range of 0.92-1.96 % in intraday precision.

In interday precision, the % recovery was observed in the range of 88.73 - 97.51 % and 89.35 - 99.63 % for azelnidipine and chlorthalidone respectively with % RSD of 1.07-2.17 % and 0.92-2.30 % respectively (Table 3). Based on the results, it can be concluded that the method was precise and accurate.

Table 1
LC–MS/MS parameters selected for the quantification of azelnidipine, chlorthalidone using nifedipine as internal standard

S.N.	Parameter	Results		
		Azelnidipine	Chlorthalidone	Nifedipine
1	Precursor ion (m/z)	583	339	347
2	Product ion (m/z)	331	321	328
3	Declustering potential (v)	49	28	40
4	Entrance potential (v)	10	10	10
5	Collision energy (v)	37	21	29
6	Cell exit potential (v)	18	13	15

Table 2
Linearity results observed in the developed method

S.N.	Peak area of azelnidipine	Peak area of chlorthalidone	Peak area of IS	Area ratio of azelnidipine and IS	Area ratio of chlorthalidone and IS
1	9895.3	7421.5	163263.8	0.061	0.045
2	16363.5	12272.6	163995.7	0.100	0.075
3	47591.4	35693.6	163024.8	0.292	0.219
4	98592.3	73944.2	163639.7	0.602	0.452
5	231153.9	183365.4	163024.8	1.418	1.125
6	485251.7	363938.8	163326.5	2.971	2.228

Table 3
Intra and interday precision and accuracy results for azelnidipine and chlorthalidone in the developed method

Analyte	QC level	Intraday precision (n=6)			Interday precision (n=6)		
		Conc. found (ng/mL)	Accuracy %	RSD %	Conc. found (ng/mL)	Accuracy %	RSD %
Azelnidipine	HQC (500 ng/mL)	495.76	99.15	1.12	481.76	96.35	0.92
	MQC (100 ng/mL)	98.75	98.75	1.11	93.30	93.30	1.10
	LQC (5 ng/mL)	4.85	96.90	1.40	4.55	90.99	1.96
Chlorthalidone	HQC (500 ng/mL)	481.52	96.30	1.49	486.05	97.21	2.20
	MQC (100 ng/mL)	100.39	100.39	1.07	95.44	95.44	0.92
	LQC (5 ng/mL)	4.87	97.40	2.17	4.62	92.44	2.30

Table 4
Different stability study results of azelnidipine and chlorthalidone in the developed method

S.N.	Test	QC level	Azelnidipine			Chlorthalidone		
			Conc. found (ng/mL)	% stability	RSD %	Conc. found (ng/mL)	% stability	RSD %
1	Short term stability	HQC (500 ng/mL)	487.55	97.51	1.06	484.80	96.96	1.11
2		MQC (100 ng/mL)	96.01	96.01	0.94	95.05	95.05	0.94
3		LQC (5 ng/mL)	4.79	95.89	1.53	4.56	91.26	1.41
4	Long term stability	HQC (500 ng/mL)	450.43	90.09	3.24	472.91	94.58	2.78
5		MQC (100 ng/mL)	91.62	91.62	3.22	88.40	88.40	1.80
6		LQC (5 ng/mL)	4.66	93.29	2.92	4.44	88.79	2.87
7	Freeze–thaw stability	HQC (500 ng/mL)	458.46	91.69	2.80	472.68	94.54	1.67
8		MQC (100 ng/mL)	93.90	93.90	2.59	98.21	98.21	1.17
9		LQC (5 ng/mL)	4.44	88.89	3.85	4.58	91.67	2.71
10	Auto-sampler stability	HQC (500 ng/mL)	482.93	96.59	1.81	469.03	93.81	1.72
11		MQC (100 ng/mL)	92.76	92.76	2.15	94.30	94.30	1.22
12		LQC (5 ng/mL)	4.55	91.05	2.99	4.58	91.52	3.79
13	Dry extract stability	HQC (500 ng/mL)	464.59	92.92	1.91	451.19	90.24	1.22
14		MQC (100 ng/mL)	90.73	90.73	2.05	89.25	89.25	2.91
15		LQC (5 ng/mL)	4.29	85.70	3.24	4.27	85.41	2.42

The recovery study confirms the efficiency of the extraction of both the analytes along with internal standard and the % recovery was calculated in three QC levels. The % recovery was observed to be in the range of 94.36 to 103.58 % for azelnidipine and 95.25 to 102.67 % for chlorthalidone. The % recovery was observed in the acceptable limit for azelnidipine and chlorthalidone in the developed method.

Hence it can be confirmed that the extraction protocol introduced by the proposed method was efficient for the separation and analysis of azelnidipine and chlorthalidone.

The matrix effect defines the efficiency of the ionization of the analytes in the ion source and whether it is affected or not by the co-eluting matrix constituents. The matrix effect was examined for azelnidipine and chlorthalidone in three QC levels. The results confirm that there was no significant matrix effect on the ionization (suppression or enhancement) of the analytes which proves that the utilized conditions for sample processing have efficaciously removed any probable interference from the matrix.

The impact of sample dilution on the accuracy and precision of the developed method was evaluated in dilution integrity study. The 2 factor higher concentration than HQC level was prepared and diluted to the HQC level prior to the analysis. The accuracy in the dilution integrity study was observed to be 97.63 % and 95.81 % respectively for azelnidipine and chlorthalidone confirming that the method was accurate and precise.

The stability studies such as short term, long term, freeze–thaw, auto-sampler and dry extract stability showed that the mean% nominal values of the analytes were within ± 15% of the predicted concentrations for the analytes at their LQC, HQC and LQC levels. The results of the stability studies were shown in table 4 confirming that the stabilities were acceptable limits and the good stability of azelnidipine and chlorthalidone proved that the method was stable.

Conclusion

A novel HPLC–MS/MS method was developed and subsequently validated for the simultaneous estimation of azelnidipine and chlorthalidone (recently approved combination for the treatment of hypertension) in spiked human plasma. The method reports a wide calibration range of 5-500 ng/mL with sensitive detection limit of 1.5 ng/mL for both azelnidipine and chlorthalidone. The method validation proved that the method shows satisfactory results in terms of its selectivity, recovery, accuracy and precision.

Various stability studies were also performed and results were in the acceptable limit. Hence it can be concluded that the method was suitable for the separation and simultaneous analysis of azelnidipine and chlorthalidone and may be applicable for the pharmacokinetic profiling of the studied drugs.

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