# A NOVEL VALIDATED BIO-ANALYTICAL METHOD DEVELOPMENT FOR AZELNIDIPINE (DIHYDROPYRIDINE) IN INDIAN HUMAN PLASMA BY LIQUID CHROMATOGRAPHY QUADRUPLE TANDEM MASS SPECTROMETRY (LC-ESI-MS/MS, API-4000) WITH AN APPLICATION TO *IN VIVO* PHARMACOKINETIC AND BIOEQUIVALENCE STUDY

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**ABSTRACT**

Azelnidipine is a dihydropyridine used as a calcium channel blocker. The main aim of this study was to develop a validated bio-analytical method (as per US-FDA and EMA guidelines) for *in vivo* pharmacokinetic and bioequivalence study of azelnidipine in human plasma by LC-MS/MS, API-4000. In this method, the drug was ionized in negative mode and gave adequate response because this drug was highly sensitive and had high electron affinity due to the presence of the electron-withdrawing the nitro group in the structure of azelnidipine. The deprotonated precursor ions $[M-H]^-$at $mz^{-1}$ 581.2 and consistent fragment ion selected was $mz^{-1}$ 491.0. For internal standard, the deprotonated precursor ions $[M-H]^-$at $mz^{-1}$ 269.0 (highest peak) was observed in Q1 MS and characteristic product ions or fragment ions found in Q3 MS were at $mz^{-1}$ 169.8. For plasma extraction, the liquid-liquid extraction technique was used. The calibration concentrated points of azelnidipine were 0.15 to 10.00 ng $mL^{-1}$ including LLOQ 0.15 ng $mL^{-1}$, LQC 0.46 ng $mL^{-1}$, MQC 3.75 ng $mL^{-1}$ and HQC 7.50 ng $mL^{-1}$. The LOD value was 0.07 ng $mL^{-1}$. The result of matrix effect of internal standard (tolbutamide) ranges between 93.51% - 98.68% and 91.94% - 95.07% for azelnidipine, recovery result after extraction of plasma of azelnidipine was 90.73% to 100.46% and for IS it was 95.95% to 98.82%. After administration of film-coated azelnidipine 8mg of test drug at 2.92±0.77h. $C_{max}$ obtained was 5.98±1.93ng $mL^{-1}$ whereas for reference drug it was 6.18±1.96ng $mL^{-1}$ $C_{max}$ at 3.03±0.98h. This method was validated as per regulatory guidelines and is highly selective, specific, highly sensitive and reproducible with low ionic suppression and high recovery, High throughput screening method was successfully applied to *in vivo* pharmacokinetic and bioequivalence study of azelnidipine.

**Keywords:** Azelnidipine, Bioequivalence study, LC-MS/MS, Dihydropyridine

## ABBREVIATIONS

USFDA: United States Food and Drug Administration; CPU: Clinical Pharmacological Unit; CDSCO: Central Drugs Standard Control Organization; ISTD: Internal standard; DMSO: Dimethyl sulfoxide; LLOQ: Lower limit of quantification; LQC: Low-quality control; MQC: Middle-quality control; HQC: High-quality control; EMA: European Medicine Agency; API:Active Pharmaceutical Ingredient; LLOD: Lower Limit of Detection; ESI: Electrospray ionization; ME: Matrix Effect; LLE: Liquid-liquid extraction; DMF: N,N-Dimethylformamide

## INTRODUCTION

Among the various dihydropyridines (calcium channel blockers) azelnidipine is one of the important drugs[1]. Azelnidipine is a benzhydrylazetidin and nitrophenyl containing dihydropyridinedicarboxylate and CAS number 123524-52-7. The chemical formula of azelnidipine is $C_{33}H_{34}N_4O_6$, molecular weight is 582.66 and monoisotopic mass is 582.24. It is a vasodilator that induces a gradual decrease in blood pressure in hypertensive patients but

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it does not induce reflex tachycardia due to vasodilation[2]. Azelnidipine has a high affinity on vascular tissue and it also has antioxidative activity, so it has strong anti-arteriosclerotic action in vessels and prolonged hypotensive effects[3]. It also reduced heart rate and proteinuria in hypertensive patients by inhibiting sympathetic nerve activity[4]. It has cardioprotective, neuroprotective, and anti-atherosclerotic properties and has preventive insulin resistance power[5]. The absorption of azelnidipine after oral administration is rapid and dose-dependent[6]. It is excreted in urine but the amount of excretion higher in feces[7]. Azelnidipine after administration undergoes primarily first-pass hepatic metabolism and is metabolized by hepatic cytochrome P450 (CYP 3A4) and has no active metabolite product[8]. It is a lipophilic drug and has a potent affinity for membranes of vascular smooth muscle cells. The biological half-life of this drug is 16-28 h. Azelnidipine inhibits trans membrane calcium ion influx through the voltage-dependent channels of smooth muscle cells in vascular walls and it is an L and T calcium channel blocker[9].

From the searching of previously published literature of bioanalytical methods of LC-MS/MS for determining azelnidipine in human plasma it was observed that it was a positively ionization method with a lengthy process and was not cost-effective. In this present study of LC-MS/MS (API-4000) for identification and quantification of azelnidipine in human plasma, a bioanalytical method has been developed as per US-FDA and EMA guideline in which drug ionized in negative mode and it is a low cost, time-consuming, and highly selective, specific, high recovery and low suppression of ionization method.

It was found that in all previous publications of bioanalytical method development of azelnidipine by LC-MS/MS, azelnidipine was ionized in positive mode but causes of ionization were not described and fragmentation of product ion was not found due to presence of nitro group in the structure which is electron withdrawing group and withdraw electron from solvent system and neutralize the positive ionization character of nitrogen present in nitro group. So, for quantitation used negative polarity to achieve adequate response for this analysis. Moreover, negative ionization mode is selective and highly sensitive for compounds with high electron affinity. Thus, negative ionization mode was selected to fragment the analyte to obtain intense and consistent product ions. Due to negative mode of azelnidipine, ionization suppression very low and high resolution, high recovery from human plasma than the positive mode. But in the present bio-analytical study azelnidipine is ionized in negative mode and causes of ionization and fragmentation of product ion of azelnidipine are described and this bio-analytical method is validated as per guidelines and also successfully applied for quantification of azelnidipine in *in vivo* pharmacokinetic and bioequivalence study.

## MATERIALS AND METHODS

### Chemical reagents

Methanol was purchased from J.T. Baker. Isopropyl alcohol, formic acid, ammonia solution DMF, dichloromethane, di-ethyl ether and ammonium acetate, were of analytical reagent grade and purchased from Merck (MERCK India Ltd., Mumbai). HPLC grade and analytical grade chemical solvents and reagents were used in the total study. Milli Q water was used in the study which was prepared from Milli-Q water purification system procured from Millipore (Elix, Milli-Q A10 Academic, and Bedford, MA, USA) until conductivity less than 0.05 μS $cm^{-1}$ was achieved. Blank human plasma was separated from EDTA-K3 containing whole blood by centrifugation and was stored at -20°C until analysis. This worked was done in Clinical Pharmacological Unit (CPU) of TAAB Biostudy Services, Kolkata.

### Ethical clearance, informed consenting of volunteers and study design

This study was carried out following clinical research guidelines for medical research involving human subjects [59th WMA General Assembly, Seoul, October 2008] and as per ICMR and Indian GCP guidelines.

This azelnidipine study protocol and informed consent form, case record form and subject information sheet were submitted to the HURIP Independent Bio-ethics committee, Kolkata, India. CDSCO registration: ECR/103/Indt/WB/2013/RR-19, valid up to 21-Nov.-2024.

All participating volunteers were voluntary informed about the study consent with written information in presence of the clinical investigator and other study team members and which was approved by the ethics committee. After written informed consent, twenty volunteers were screened and eighteen volunteers were included in this study. All participating volunteers were randomized and their rights and health protection were ensured by the human ethics committee and study team.

This study was a comparative pharmacokinetic study and all volunteers received both test and reference film-coated tablets containing azelnidipine

8 mg only on the study day at a fixed time. The washout period of azelnidipine was 7 days between the two dosing sessions.

**Table I: Randomization schedule**

| Subject No. | Period I | Period II |
|---|---|---|
| 1 | *A1 | *A2 |
| 2 | *A2 | *A1 |
| 3 | *A2 | *A1 |
| 4 | *A1 | *A2 |
| 5 | *A1 | *A2 |
| 6 | *A2 | *A1 |
| 7 | *A1 | *A2 |
| 8 | *A2 | *A1 |
| 9 | *A2 | *A1 |
| 10 | *A1 | *A2 |
| 11 | *A1 | *A2 |
| 12 | *A2 | *A1 |
| 13 | *A1 | *A2 |
| 14 | *A2 | *A1 |
| 15 | *A2 | *A1 |
| 16 | *A1 | *A2 |
| 17 | *A1 | *A2 |
| 18 | *A2 | *A1 |

**A1 – Reference Preparation and *A2 – Test Preparation*

## Study protocol

This study was a single-dose, randomized, open-label, two treatment, and two–way cross-over bio-equivalence study. It was a comparative pharmacokinetic study of a film-coated tablet containing azelnidipine 8 mg as test preparation and reference preparation (Uniaz 8 tablet manufactured by M/s Precise Chemipharma Pvt. Ltd. Maharashtra, India.) in eighteen healthy, adult, human, male volunteers in a fasting condition. The randomization schedule of this study is illustrated in Table I.

During oral administration of test and reference tablets, each volunteer was administered 240±2.0 mL water with a tablet in sitting posture after an overnight fast of at least 10 h. Lunch, snacks, and dinner were provided after 4 h, 8 h and 14 h, respectively, after drug ingestion. On the study days, volunteers were permitted normal activities, excluding strenuous exercise. A total of seventeen blood samples of each volunteer were collected at 0 h. (before drug administration) and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 8.0, 12.0, 24.0, 48.0 and 72.0h in EDTA containing vials. The mean BMI of eighteen volunteers was 21.28 kg $m^{-2}$. The BMI of each volunteer is specified in Table II. For calculation of pharmacokinetic parameters of azelnidipine, SAS software was used.

## Bioanalytical method development (gradient method) of azelnidipine in human plasma by LC-ESI-MS/MS(API-4000)

Azelnidipine is a benzhydrylazetid in and nitrophenyl containing dihydropyridine dicarboxylate compound as illustrated in Fig. 1. The plasma protein binding of azelnidipine 90-91%. The naming of azelnidipine is due to the presence of benzhydrylazetidin-3-ol which has pKa value 4.59 to 4.79, hence acidic. The long-lasting action of azelnidipine is due to the presence of 3-nitrophenyl in the structure, which is slightly acidic in character and with pKa value 8.36. The nitro group is a strongly electron-withdrawing group and it can be acidic due to the adjacent alpha C–H bonds of the nitro group. In azelnidipine there is a dihydropyridine due to the presence of 2-amino-3-methyl-dihydropyridine which has pKa value 3-5.23 hence so acidic. On the other hand, it contains one isopropyl alcohol which has pKa value 17.1 that is strongly basic

**Table II: BMI of each volunteer**

| Subject No. | Sex | Age | Height (cm) | Weight (kg) | BMI (kg $m^{-2}$) |
|---|---|---|---|---|---|
| 1 | M | 25 | 155 | 57 | 23.73 |
| 2 | M | 35 | 166 | 55 | 19.96 |
| 3 | M | 39 | 163 | 53 | 19.95 |
| 4 | M | 41 | 169 | 56 | 19.61 |
| 5 | M | 23 | 171 | 55 | 18.81 |
| 6 | M | 28 | 162 | 55 | 20.96 |
| 7 | M | 34 | 162 | 55 | 20.96 |
| 8 | M | 27 | 170 | 60 | 20.76 |
| 9 | M | 45 | 165 | 55 | 20.2 |
| 10 | M | 37 | 165 | 62 | 22.77 |
| 11 | M | 32 | 157 | 55 | 22.31 |
| 12 | M | 34 | 170 | 55 | 19.03 |
| 13 | M | 39 | 155 | 60 | 24.97 |
| 14 | M | 29 | 163 | 64 | 24.09 |
| 15 | M | 43 | 161 | 55 | 21.22 |
| 16 | M | 26 | 160 | 59 | 23.05 |
| 17 | M | 26 | 165 | 60 | 22.04 |
| 18 | M | 42 | 170 | 54 | 18.69 |
| Mean | | 33.61 | 163.83 | 56.94 | 21.28 |
| S.D. | | 7.03 | 4.92 | 2.97 | 1.81 |

and two carboxylic acid groups that form dicarboxylate, which is also acidic in characters. So overall compound pKa value is 19.88, that is strongest acidic. The exact mass of azelnidipine is 582.24 (molecular wt. 582.66). Due to the presence of the nitro group in the structure which is an electron-withdrawing group, it withdraws electron from the solvent system and neutralizes the positive ionization character of nitrogen present in the nitro group. So, quantitation method used negative polarity to achieve an adequate response for this analysis. Due to high electron affinity, negative ionization mode was selected for ionization of azelnidipine. Thus, negative ionization mode was selected to fragment the analyte to obtain intense and consistent product ions.

The deprotonated precursor ions $[M-H]^-$ at $mz^{-1}$ 581.2 (highest peak), 583.2 (2nd peak) and 591.2 (3rd peak), were observed in Q1 MS in which selected parent ion 581.2 for azelnidipine and characteristic product ions or fragment ions found in Q3 MS were at $mz^{-1}$ 491.0,534.7. However, the most stable and consistent fragment ion selected was $mz^{-1}$ 491.0 [(M-$C_4H_{10}O_2$-H) -] for releasing 1,2-butanediol, which is represented in Fig. 2.

Tolbutamide was used as an internal standard. The deprotonated precursor ions $[M-H]^-$ at $mz^{-1}$ 269.0

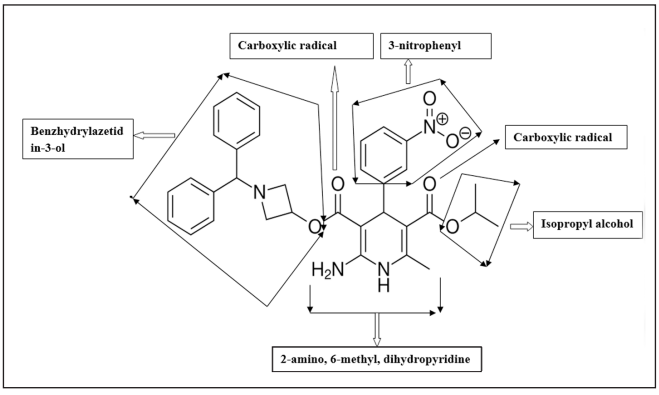


**Fig. 1: Chemical structure of azelnidipine**

(highest peak), was observed in Q1 MS and characteristic product ions or fragment ions found in Q3 MS were at $mz^{-1}$ 169.8.

The chromatographic elution of the analyte on a Phenomenex Kinetex 5µ C18 100A 50*3mm column was initiated as a rapid, highly sensitive, and rugged analytical method covering the dynamic linear range. Mobile phase selection was necessary for the analysis of the drug depending on its pKa value. Thus, the pH of the mobile phase, buffer concentration, and choice and proportion of diluents were very important for chromatographic resolution with adequate response to achieve the desired

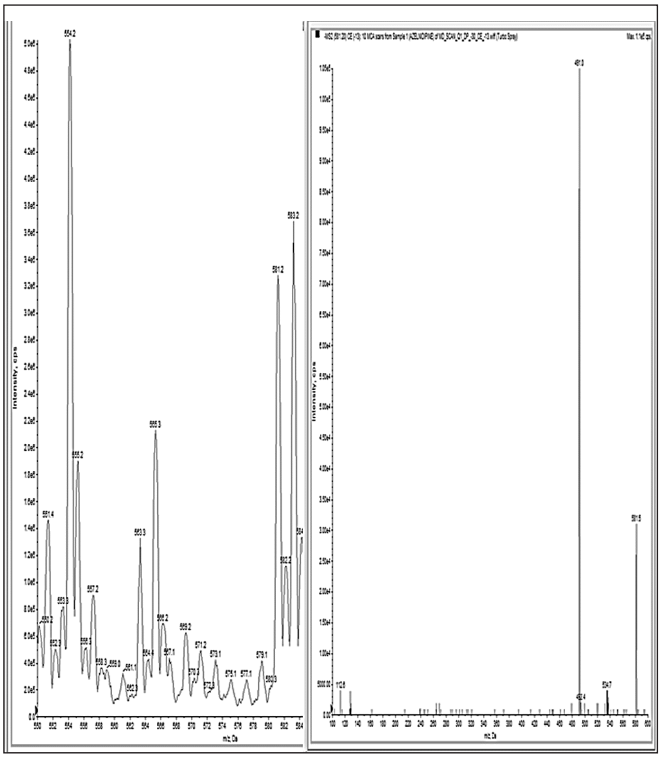

**Fig. 2: Parent ion (Q1) and product ion (Q3) scan of azelnidipine**

sensitivity. Optimized instrumental (mass) parameters for the analyte and IS are illustrated in Table III.

Initially, acetonitrile/methanol with 1 mM ammonium acetate buffer (pH 6.5) gave a response for azelnidipine. However, the response was not reproducible. The signal of the lower limit of quantification concentration was changed when buffer concentration increased from 1mM to 10mM. Further, the chromatography was better with a higher response using a methanol-buffer as compared to acetonitrile-buffer combination. Moreover, a higher concentration of the methanol content in the mobile phase resulted in an increase in the retention of azelnidipine and thereby the analysis time. Subsequent efforts were directed to optimize the pH of the mobile phase and the concentration of the buffer solution as they had a significant impact on analyte retention, peak shape, and resolution. At pH above 5.0, the resolution of azelnidipine was affected, which further deteriorated with an increase in pH. Thus, to achieve greater reproducibility and better chromatography, low pH buffers were tried. Reproducibility and peak shape of the lower concentration of the drug was better in 0.1% formic acid but the signal-noise ratio was not adequate. A superior signal to noise ratio ($\geq$ 22) and baseline resolution was obtained for the analyte by 20 mM ammonium acetate buffer with 1.0% (V/V) formic acid together with Milli Q water having apparent pH

**Table III: Optimized instrumental (mass) parameters for azelnidipine and IS**

| Parameter | Value |
|---|---|
| Ionization mode | MRM (-ve) |
| Source temperature (°C) | 400 |
| Dwell time per transition (msec) | 100 |
| Curtain gas (psi) | 30 |
| CAD gas (psi) | 8 |
| Ion spray voltage (V) | -4500.00 |
| Ion source gas 1 (psi) | 40 |
| Ion source gas 2 (psi) | 40 |
| Focusing potential (V) | 400 |
| Declustering potential (V) | -30 (azelnidipine) and -30 (IS) |
| Entrance potential (V) | -10 |
| Collision energy (V) | -13 (azelnidipine) and -27 (IS) |
| Collision cell exit potential (V) | -15 ( azelnidipine and IS) |
| Transition pair of azelnidipine (analyte) | 581.2/491.0 |
| Transition pair of tolbutamide (IS) | 269.0/169.8 |

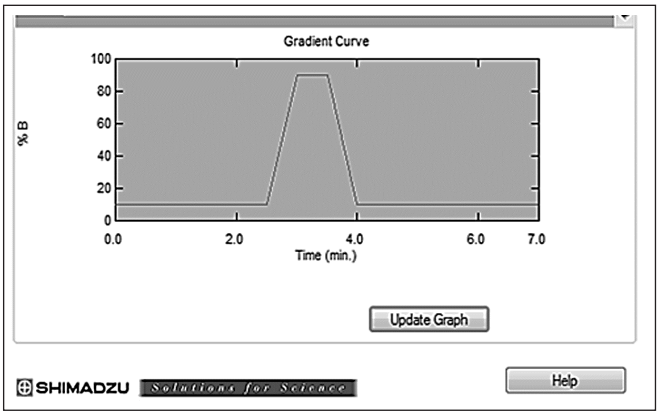


**Fig. 3: Gradient curve of method development of azelnidipine**

2.10 at a flow rate of 0.5000 mL $min^{-1}$. Here, formic acid improves the separation and increases the MS sensitivity and the analyte could be separated from the endogenous substances in the plasma. But organic solvent methanol contains 1.0% ammonia solution because of electron withdraw from this solvent by the nitro group present in azelnidipine. As a result, suppression of negative ionization of parent compound was less and responded in high intensity.

In the present study, the chromatographic part was performed by gradation method in which 10 % organic solvent was used for 0.01 min to 3.00 min and 90% organic solvent used for 3.00 min to 4.00 min of total run time, whereas 90 % aqueous solvent was used from 4.00 min to rest of the total run time (7.00min) for washing purpose. The gradient curve is shown in Fig.3. The chromatographic elution time for azelnidipine was found 4.61 min and for IS it was 4.33 min. in a total run time of 7.00 min. The representative MRM chromatograms are shown in Figs. 4a, 4b, 4c, 4d, 4e and 4f.

## Plasma extraction and sample preparation

### Plasma extraction procedure

Plasma extraction was performed by the liquid-liquid extraction technique. 400 µL volume of plasma sample was transferred to a 15 mL plastic Tarson tube, and then 100 µL of internal standard tolbutamide was spiked for getting 1µg $mL^{-1}$ of internal standard concentration in the final prepared sample. 2.0 mL DMF was added and then vortexed 5 min. 2.5 mL of the mixture (1:3) of dichloromethane and di-ethyl ether was added to the sample tubes. After that, the sample was vortex-mixed for 5min and then centrifuged at 5000 rpm for 10 min. Thus obtained supernatant organic layer (3.5 mL) was transferred to a 15 mL plastic Tarson tube and evaporated to dryness at 40°C under a stream of nitrogen. Then the dried extract was reconstituted in 200 µL of diluents (mobile phase) methanol: water (50:50 V/V) and vortexed for 2 min then was taken into autosampler vial and 10µL aliquot was injected into chromatographic system.

## Stock solution and calibration standards preparation

### Preparation of azelnidipine stock solution (w/V)

1.0 mg of azelnidipine was accurately weighed and dissolved in 1.0mL DMSO, then mixed well and vortexed. The final concentration of azelnidipine was 1mg $mL^{-1}$. This stock solution was used for the preparation of an intermediate concentration of azelnidipine. It was stored in a refrigerator at 2-8°C.

### Preparation of tolbutamide (ISTD) stock solution (w/V)

1.0 mg of tolbutamide was accurately weighed and dissolved in 1.0mL DMSO, then mixed well and vortexed. The final concentration of tolbutamide was 1mg $mL^{-1}$. This stock solution was used for the preparation of an intermediate concentration of tolbutamide. It was stored in a refrigerator at 2-8°C.

**Table IVa: Pre-study linearity of detector response (n=3)**

| Linearity | Concentration (ng mL$^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.15 | 0.31 | 0.62 | 1.25 | 2.5 | 5 | 10 |
| LIN 1 | 0.15 | 0.33 | 0.58 | 1.13 | 2.37 | 5.05 | 11.31 |
| LIN 2 | 0.17 | 0.33 | 0.59 | 1.24 | 2.26 | 4.83 | 11.28 |
| LIN 3 | 0.14 | 0.32 | 0.65 | 1.07 | 2.47 | 5 | 10.88 |
| Average | 0.153 | 0.327 | 0.607 | 1.147 | 2.367 | 4.96 | 11.157 |
| S.D | 0.015 | 0.006 | 0.038 | 0.086 | 0.105 | 0.115 | 0.24 |
| % C.V. | 9.962 | 1.767 | 6.241 | 7.519 | 4.438 | 2.325 | 2.152 |
| Nominal % | 102.22 | 105.38 | 97.85 | 91.73 | 94.67 | 99.2 | 111.57 |

**Table IV b: Pre-study linearity of detector response statistics (n=3)**

| Linearity | Statistics | | |
|---|---|---|---|
| **LINEARITY CODE** | **SLOPE (m)** | **INTERCEPT (c)** | **R square** |
| LIN 1 | 0.00042 | 0.00002 | 0.9953 |
| LIN 2 | 0.00038 | 0.00004 | 0.9958 |
| LIN 3 | 0.0007 | 0.00007 | 0.996 |
| MEAN | 0.00023 | NOT APPLICABLE | 0.9959 |
| S.D. | 0.00022 | | 0.00014 |
| C.V.% | 95.22 | | 0.014 |

**Table V: Precision and accuracy (n = 5)**

| | Between run | | | Within run | | |
|---|---|---|---|---|---|---|
| | **Mean ± SD** | **C.V.%** | **Absolute bias (%)** | **Mean ± SD** | **C.V.%** | **Absolute bias (%)** |
| LLOQ (0.15 ng mL$^{-1}$) | 0.153±0.008 | 5.232 | 101.78 | 0.150±0.010 | 6.667 | 100.01 |
| LQC (0.46 ng mL$^{-1}$) | 0.455±0.037 | 8.182 | 96.74 | 0.474±0.052 | 11.023 | 100.85 |
| MQC (3.75 ng mL$^{-1}$) | 3.659±0.319 | 8.714 | 97.58 | 3.822±0.226 | 5.904 | 101.92 |
| HQC (7.50 ng mL$^{-1}$) | 7.541±0.632 | 8.381 | 100.54 | 7.868±0.335 | 4.253 | 104.91 |

## Preparation of calibration concentrations in plasma

Prepared stock solutions of azelnidipine and IS (tolbutamide) diluted with methanol: water 50: 50 (V/V) and intermediate concentrations prepared. Each of 500 µL of the corresponding concentrated solutions of azelnidipine were transfered into 500 µL blank human plasma to achieve calibration the concentrated points 0.15, 0.31, 0.62, 1.25, 2.50, 5.00 and 10.00 ng mL$^{-1}$, including LLOQ 0.15 ng mL$^{-1}$, LQC 0.46 ng mL$^{-1}$, MQC 3.75 ng mL$^{-1}$ and HQC 7.50 ng mL$^{-1}$.

## Bio-analytical method validation of developed method[10-18]

The method validation of azelnidipine was conducted following the guidelines US-FDA and EMA for selectivity, sensitivity, linearity, precision, accuracy, recovery, and stability.

## Specificity, selectivity and linearity

Specificity is the ability of the bioanalytical method to differentiate and quantify the analyte in a complex mixture, that is, it is the method's ability responding to one single

**Table VI: Stability Study (Freeze thaw, Short term, Auto sampler, Bench top stability, Long term stability)**

| Study | | Inj No. | LQC (0.46 ng $mL^{-1}$) | MQC (3.75 ng $mL^{-1}$) | HQC (7.50 ng $mL^{-1}$) |
|---|---|---|---|---|---|
| | Freshly Thawed | 1 | 0.43 | 3.21 | 8.16 |
| | | 2 | 0.42 | 3.95 | 7.57 |
| | | 3 | 0.47 | 4.20 | 8.07 |
| | | 4 | 0.46 | 3.45 | 8.50 |
| | | 5 | 0.43 | 3.79 | 6.91 |
| | | Mean | **0.44** | **3.72** | **7.84** |
| **Freeze thaw stability** | After 3 cycle | 1 | 0.43 | 3.52 | 8.54 |
| | | 2 | 0.45 | 4.13 | 8.12 |
| | | 3 | 0.5 | 3.93 | 8.57 |
| | | 4 | 0.45 | 3.88 | 7.55 |
| | | 5 | 0.47 | 3.88 | 7.95 |
| | | Mean | **0.46** | **3.87** | **8.15** |
| | % Stability | | **104.07** | **103.98** | **103.88** |
| **Short term stability** | After 24 h | 1 | 0.43 | 3.28 | 7.7 |
| | | 2 | 0.42 | 3.64 | 8.33 |
| | | 3 | 0.49 | 3.85 | 7.6 |
| | | 4 | 0.43 | 3.9 | 8.31 |
| | | 5 | 0.42 | 3.6 | 6.75 |
| | | Mean | **0.44** | **3.65** | **7.74** |
| | % Stability | | **99.10** | **98.23** | **98.67** |
| **Auto sampler stability** | After 24 h in auto sampler (15°C) | 1 | 0.47 | 3.48 | 7.81 |
| | | 2 | 0.46 | 3.9 | 7.35 |
| | | 3 | 0.47 | 3.73 | 7.16 |
| | | 4 | 0.50 | 3.58 | 7.74 |
| | | 5 | 0.44 | 3.61 | 7.93 |
| | | Mean | **0.47** | **3.66** | **7.60** |
| | % Stability | | **105.88** | **98.39** | **96.89** |
| **Bench top stability** | After 24 h in laboratory room temperature | 1 | 0.46 | 3.42 | 7.69 |
| | | 2 | 0.49 | 3.54 | 6.99 |
| | | 3 | 0.42 | 3.48 | 8.61 |
| | | 4 | 0.42 | 3.59 | 6.90 |
| | | 5 | 0.43 | 3.61 | 6.75 |
| | | Mean | **0.44** | **3.53** | **7.39** |
| | % Stability | | **100.45** | **94.84** | **94.21** |
| **Long term stability** | | 1 | 0.45 | 3.43 | 8.06 |
| | | 2 | 0.49 | 3.90 | 7.70 |
| | | 3 | 0.51 | 4.02 | 6.96 |
| | | 4 | 0.51 | 3.97 | 7.19 |
| | | 5 | 0.46 | 4.14 | 6.85 |
| | | Mean | **0.48** | **3.89** | **7.35** |
| | % Stability | | **109.50** | **104.62** | **93.75** |

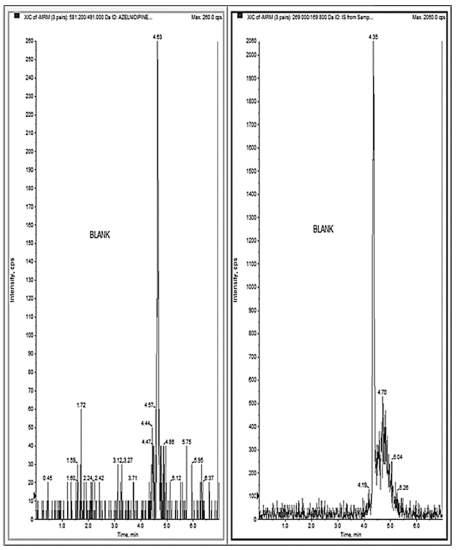


**Fig. 4a: Blank sample chromatogram of azelnidipine**

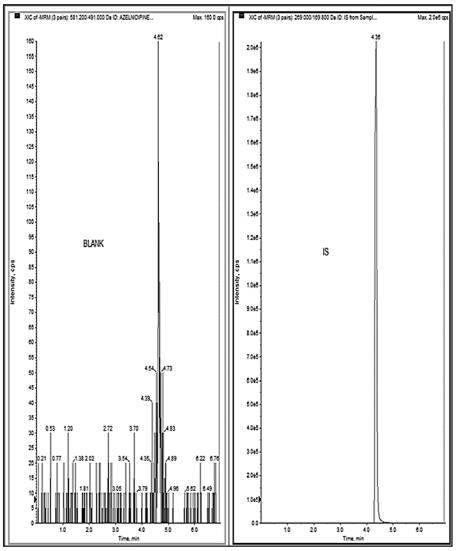


**Fig. 4b: Blank + IS sample chromatogram of azelnidipine**

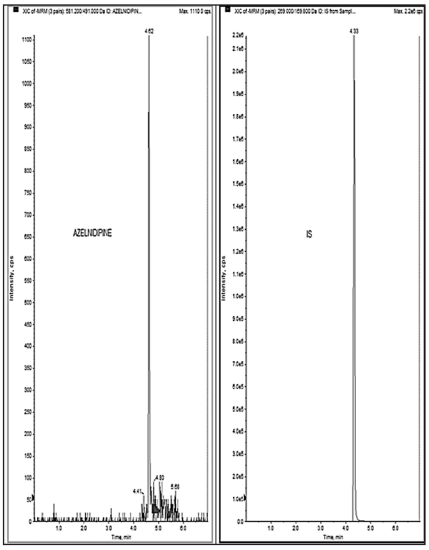


**Fig. 4c: LLOQ sample chromatogram of azelnidipine**

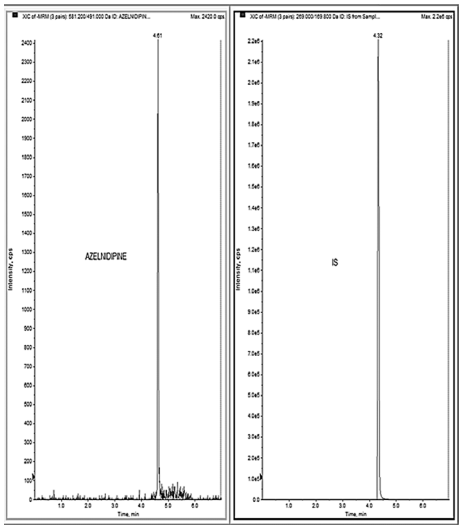


**Fig. 4d: LQC sample chromatogram of azelnidipine**

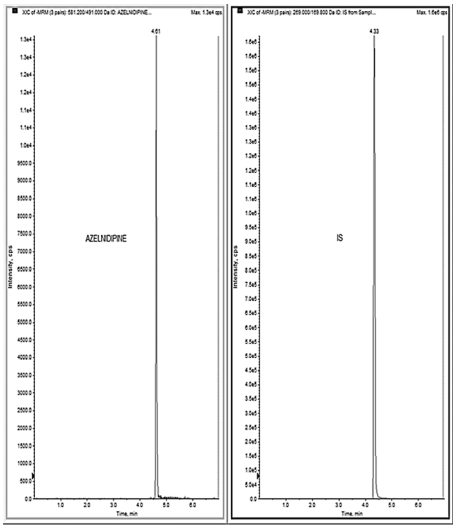


**Fig. 4e: MQC sample chromatogram of azelnidipine**

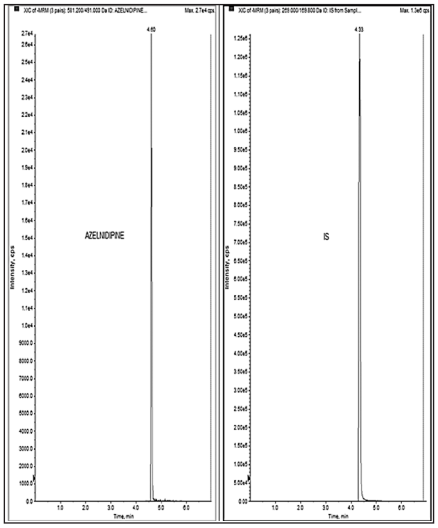


**Fig. 4f: HQC sample chromatogram of azelnidipine**

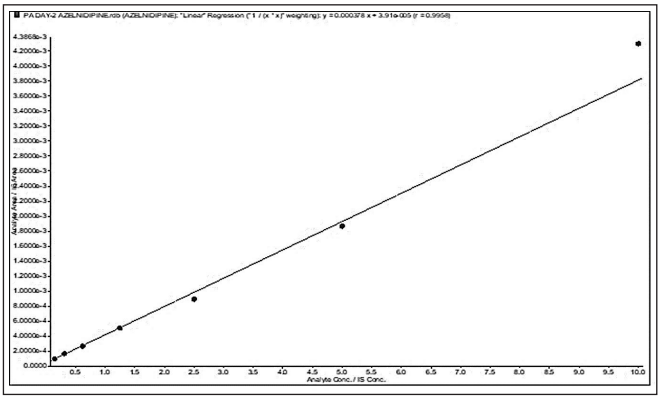

**Fig. 5: Plasma calibration curve of azelnidipine**

analyte only but the selectivity of a bioanalytical method is the ability of the method to differentiate and quantify the analyte in the presence of other components in the sample. These two were described by intensity and wide of the chromatograms of LLOQ of the analyte. The linearity of the method was described by the nominal percentage of the calibration concentrations of three consecutive days. The mean regression value of the three linearities were calculated by the intercept and slope of the three-calibration curve of the respective days. The calibration curve was always linear and was described by a linearity graph.

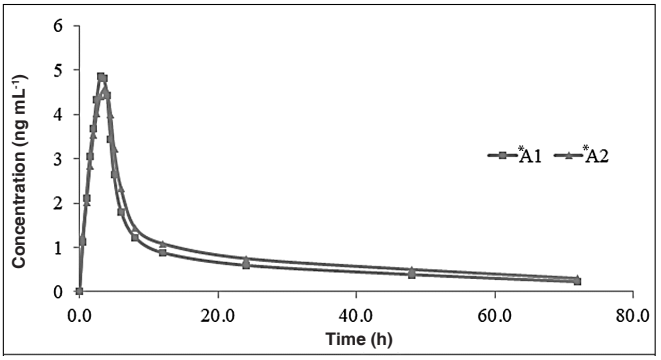


**Fig. 6: Mean (eighteen volunteers) comparative plasma concentration-time pharmacokinetic graph of azelnidipine (8mg)**

### Precision and accuracy

The within–batch accuracy and precision and between–batch accuracy and precision were assessed by the repeated analysis of blood samples containing different concentrations of LQC, MQC, and HQC on three precision and accuracy batches using analyte on separate occasions (n=5). Total calibration curve points, HQC, MQC, and LQC samples should be within ±15% of the specified concentrations, except for the LLOQ within ±20%. Precision and accuracy are determined by % CV which is within15% at HQC, MQC, LQC, and LLOQ QC levels.

### Stability

This bioanalytical method was validated as per US-FDA and EMA guidelines. The stability parameters like short term, freeze-thaw, autosampler, benchtop, and long-term stability were performed as per US-FDA and EMA guidelines. As per guidelines, short term stability should be within 90-110%, freeze-thaw stability should be within 85-115%, autosampler stability should be within 85-115%, benchtop stability should be within 90-110%, and long-term stability should be within 90-110%.

### Matrix effect and recovery

In this present study, matrix effect and recovery were assayed by comparing the peak area of the analyte and IS of extracted plasma with an unextracted sample of the same analyte and IS of the same concentrations of LQC, MQC, and HQC. As per guidelines, matrix effect should be within 85-115% and recovery should be within 80-120%.

## RESULT AND DISCUSSION

Result of bioanalytical method validation[13-18]

### Specificity, selectivity and linearity

This study was done by linearity 1, 2 and 3 which were found linear. The representative calibration curve is shown in Fig. 5, which was linear between 0.15-10 ng $mL^{-1}$ ranges of calibration concentrations. Pre-study linearity of detector response with statistics is also represented in Table IVa and Table IVb. The mean regression value was linear and it was 0.9959.The nominal percentage or accuracy of calibration concentrated samples 0.15-10 ng $mL^{-1}$ was 91.73-111.57%. The LLOQ value was 0.15 ng $mL^{-1}$ and the LOD value was 0.07 ng $mL^{-1}$.

### Precision and accuracy

The percentage of the coefficient of variation result of between–run precision values ranged from 5.232% to 8.714% and absolute percent bias values were 101.78% for LLOQ, 96.74% for LQC, 97.58% for MQC and 100.54% for HQC samples.

The percentage of the coefficient of variation result of within-run precision values ranged from 4.253% to 11.023% and absolute percent bias values were 100.01% for LLOQ, 100.85% for LQC, 101.92% for MQC, and 104.91% for HQC samples. The result of precision and accuracy are elaborated in Table V.

### Stability

The freeze-thaw, short term, autosampler, benchtop, long term stability study data are elaborated in Table VI.

### Freeze-thaw stability

The freeze-thaw stability of azelnidipine ranges was between 103.88 % - 104.07 % after three cycles.

**Table VII: Matrix effect (area) (N = 5)**

| | Matrix effect IS | | Matrix effect azelnidipine | |
|---|---|---|---|---|
| | % of ME | Matrix factor | % of ME | Matrix factor |
| LQC (0.46 ng $mL^{-1}$) | 93.51± 2.59 | 0.93± 0.02 | 91.94± 2.46 | 0.91± 0.02 |
| MQC (3.75 ng $mL^{-1}$) | 98.68± 0.72 | 0.99± 0.01 | 95.07± 3.05 | 0.94± 0.04 |
| HQC (7.50 ng $mL^{-1}$) | 96.60± 1.44 | 0.97± 0.01 | 94.86± 4.50 | 0.95± 0.05 |

**Table VIIIa: Recovery of IS**

| Inj No. | Diluent Sample | | | In Plasma | | |
|---|---|---|---|---|---|---|
| | LQC | MQC | HQC | LQC | MQC | HQC |
| | 0.46 ng $mL^{-1}$ | 3.75 ng $mL^{-1}$ | 7.50 ng $mL^{-1}$ | 0.46 ng $mL^{-1}$ | 3.75 ng $mL^{-1}$ | 7.50 ng $mL^{-1}$ |
| 1 | 8440309 | 6833216 | 6337445 | 8228346 | 6958113 | 6244308 |
| 2 | 8462790 | 6315940 | 6382822 | 8099742 | 6747168 | 6142352 |
| 3 | 8161643 | 7377560 | 6003729 | 7946169 | 6861261 | 5700012 |
| 4 | 8327920 | 7047167 | 6327201 | 8328865 | 6785699 | 5882838 |
| 5 | 8725576 | 7217246 | 5592237 | 7809066 | 7027716 | 5969637 |
| Mean | 8423647 | 6958226 | 6128687 | 8082437 | 6875991 | 5987829 |
| % Recovery | | | | 95.95 | 98.82 | 97.7 |

**Table VIIIb: Recovery of azelnidipine**

| Inj. No. | Diluent Sample | | | In Plasma | | |
|---|---|---|---|---|---|---|
| | LQC | MQC | HQC | LQC | MQC | HQC |
| | 0.46 ng $mL^{-1}$ | 3.75 ng $mL^{-1}$ | 7.50 ng $mL^{-1}$ | 0.46 ng $mL^{-1}$ | 3.75 ng $mL^{-1}$ | 7.50 ng $mL^{-1}$ |
| 1 | 2994.06 | 13273.02 | 26722.03 | 2995.86 | 13175.92 | 24188.68 |
| 2 | 2726.34 | 13851.73 | 24632.45 | 2702.29 | 11474.85 | 21423.16 |
| 3 | 2920.78 | 14165.62 | 22677.5 | 2718.48 | 13738.18 | 22054.81 |
| 4 | 2914.53 | 13213.12 | 23390.44 | 3025.68 | 12594.43 | 23313.79 |
| 5 | 2770.48 | 14327.87 | 21733.34 | 2950 | 11464.22 | 20385.38 |
| Mean | 2865.24 | 13766.27 | 23831.15 | 2878.46 | 12489.52 | 22273.16 |
| % Recovery | | | | 100.46 | 90.73 | 93.46 |

### Short term stability

The stability of low, medium and high-quality control samples was determined after keeping the samples in the freeze (2-8°C) for 24 h and comparing them against fresh plasma samples of the same concentration. The short-term stability of azelnidipine ranges was between 98.23 % to 99.10 %.

### Autosampler stability

The autosampler stability of low, medium and high-quality control samples was determined by comparing freshly prepared plasma samples QC against samples QC kept in an autosampler at 15°C for 24 h. The autosampler stability of azelnidipine has resulted in values of 96.89 % to 105.88 %.

### Benchtop stability

The QC samples of plasma were kept for 24 h at room temperature on a sample preparation bench and then processed and analyzed and compared with freshly prepared plasma sample. Percentage stability was within 94.21 % to 100.45 % for azelnidipine.

### Long term stability

The long-term stability of low, medium, and high-quality control samples was determined by comparing fresh plasma samples of the QC concentrations against plasma samples of the same QC concentrations prepared and frozen 5 days earlier. The long-term stability of azelnidipine was in 93.75% to 109.50% range.

**Table IX: Mean comparative plasma pharmacokinetic profile of azelnidipine (8mg)**

| Pharmacokinetic parameters | | Azelnidipine | |
|---|---|---|---|
| | | Reference Preparation (A1) | Test Preparation (A2) |
| $C_{max}$ (ng $mL^{-1}$) | Mean | 6.18 | 5.98 |
| | ±Σ.Δ. | 1.96 | 1.93 |
| $T_{max}$ (h) | Mean | 3.03 | 2.92 |
| | ±Σ.Δ. | 0.98 | 0.77 |
| AUC 0-t (ngh $mL^{-1}$) | Mean | 63.21 | 60.62 |
| | ±Σ.Δ. | 32.9 | 27.75 |
| AUC 0-inf (ngh $mL^{-1}$) | Mean | 71.14 | 66.21 |
| | ±Σ.Δ. | 38.2 | 30.91 |
| $K_{el}$ ($h^{-1}$) | Mean | 0.041 | 0.043 |
| | ±Σ.Δ. | 0.007 | 0.007 |
| $T_{1/2}$ (h) | Mean | 17.52 | 16.49 |
| | ±Σ.Δ. | 3.08 | 3.12 |
| Relative Bioavailability (%) | | 100% | 95.90% |

### Matrix effect

The result of matrix effect of internal standard (tolbutamide) ranges between 93.51 % - 98.68 % and 91.94 % - 95.07 % for azelnidipine, and this was determined by measuring the peak areas of the drug and IS from the prepared plasma of low, medium, and high-quality control concentrations. The peak areas of the plasma low, medium, and high-quality control samples were compared to the absolute peak areas of the unextracted samples containing the same concentrations of the azelnidipine and IS. The matrix factor ranges of azelnidipine were 0.91 to 0.95 and for IS it was 0.93 to 0.99. The results of the matrix effect are elaborated in Table VII.

### Recovery

The nitro group in the structure, is an electron withdrawing group and withdraws electron from solvent system and neutralizes the positive ionization character of nitrogen present in nitro group. So, for quantitation negative polarity was used to achieve adequate response for this analysis. Moreover, negative ionization mode was selective and is highly sensitive for compounds with high electron affinity. Thus, negative ionization mode was selected to fragment the analyte to obtain intense and consistent product ions. In this method, applied collision energy and declustering potential, usable organic solvent was very low than positive method and low ionic suppression and also higher recovery after extraction of plasma of azelnidipine in negative method than positive method.

The recovery result after extraction of plasma of azelnidipine was 90.73% to 100.46% and for IS it was 95.95% to 98.82%. The percentage recoveries were determined by measuring the peak areas of the drug from the prepared plasma low, medium, and high-quality control samples. The peak areas of the low, medium and high-quality control concentrated plasma samples were compared to the absolute peak area of the unextracted samples containing the same concentrations of the IS and azelnidipine. The results of recovery are elaborated in Table VIIIa and Table VIIIb.

## RESULT OF COMPARATIVE PHARMACOKINETIC STUDY

From the analysis of eighteen volunteers' unknown blood plasma samples of test and reference of azelnidipine 8mg tablet at the different intervals, it was observed that after administration of test and reference preparation of single-dose azelnidipine in the fasting state, the maximum plasma concentration ($C_{max}$) of azelnidipine of test preparation was 5.98±1.93 ng $mL^{-1}$ at 2.92±0.77 h. ($T_{max}$) which in the case of reference preparation it was 6.18±1.96 ng $mL^{-1}$ at 3.03±0.98 h($T_{max}$). The $AUC_{0-t}$ result for test preparation obtained was 60.62 ± 27.75ng h $mL^{-1}$ and for reference preparation, it was 63.21±32.90 ng h $mL^{-1}$. The $AUC_{0-\infty}$ result for test preparation obtained was 66.21±30.91 n gh $mL^{-1}$ and for reference preparation, it was 71.14±38.20 n gh $mL^{-1}$. The elimination constant ($K_{el}$) for test preparation was 0.043±0.007$h^{-1}$ and for reference preparation, it was 0.041±0.007 $h^{-1}$. The plasma elimination half life of test preparation was 16.49±3.12h and for reference preparation plasma elimination half-life was 17.52±3.08h. The mean comparative plasma concentration-time graph and profile of azelnidipine are illustrated in Fig. 6 and Table IX.

## DISCUSSION

From this bio-analytical study of azelnidipine 8mg in human plasma by LC-MS/MS, API-4000, it was observed that this bio-analytical method was highly specific, sensitive and reproducible with high recovery result, low ionic suppression and all stability results were within acceptable limits according to guidelines. So, this

method was validated according to guidelines. In this method, drug azelnidipine was ionized in negative mode and this method was successfully applied in an *in vivo* pharmacokinetic and bioequivalence study. Different excipients present in test and reference preparation did not affect the suitability of method because of high resolution of azelnidipine due to negative mode of ionization. So, this method is highly suitable. From the results of comparative pharmacokinetic parameter $AUC_{0-t}$ of azelnidipine 8mg, it was observed that after single-dose administration of azelnidipine, the relative bioavailability of azelnidipine test preparation was 95.90% in comparison with the reference preparation. For $C_{max}$, ln $C_{max}$, $AUC_{0-t}$ and Ln $AUC_{0-t}$ value calculation, statistical ANOVA test (subject, period, treatment) was applied and it was observed that there was no statistically significant difference for the treatment values of $C_{max}$, Ln $C_{max}$, $AUC_{0-t}$, and Ln $AUC_{0-t}$, 90% confidence interval for $C_{max}$, Ln $C_{max}$, $AUC_{0-t}$, and Ln $AUC_{0-t}$ values of test preparation were within the acceptable limits of that of the reference preparation (i.e., 0.8 – 1.2).

### Safety assessment

This drug was already approved by CDSCO and previously many researchers worked on the pharmacokinetic and pharmacodynamic study of this drug. From the literature of clinical study, it was observed that only 14.4% of cases reported side effects among more than 1100 cases getting approval and most of the side effects showed up in more than 65 years old people. But in this study, clinical examination was done at the time of screening and vital signs like blood pressure, heartbeat, pulse rate, and liver function test were done before the study and 3, 6, 9, and 12 h post-dose and always checking under medical doctors. Any adverse reaction has not been complained about during the total study period.

## CONCLUSION

The present study of bioanalytical method development for identification and quantification of azelnidipine was a new ionization mode that was unique from previous publications. In this method, the drug was ionized in the negative mode because of the presence of the nitro group in the structure of azelnidipine. Plasma is extracted by liquid-liquid extraction technique. This LLE technique used DMF as a plasma extraction reagent and another solvent used was diethyl ether and dichloromethane in a ratio as an ultimate solvent for extraction of the drug. This method was validated as per US-FDA and EMA guidelines. This LC-MS/MS (API-4000) bio-analytical method of azelnidipine was highly selective, specific, highly sensitive, and reproducible with low ionic suppression, high recovery, and high throughput screening. The present method was successfully applied to the pharmacokinetic and bioequivalence studies.


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