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High Performance Liquid Chromatographic Method Development and its Validation for Lacidipine

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Abstract: A simple and sensitive high performance liquid chromatographic (HPLC) method was developed for quantification of lacidipine in human plasma. Felodipine was used as an internal standard (IS). The present method used protein precipitating extraction of lacidipine from plasma. Separation was carried out on reversed-phase c_{18} column (250 mm × 4.6 mm, 5µ) and the column effluent was monitored by UV detector at 320 nm. The mobile phase used was methanol: 0.5 mm ammonium acetate (ph 4.0), (70: 30 % v/v) at a flow rate of 1.0 mL/min. This method was linear over the range of 25.0 – 150.0 ng/mL with regression coefficient greater than 0.99. The method was found to be precise, accurate and specific during the study. The simplicity of the method allows for application in laboratories that lack sophisticated analytical instruments such as LC–MS/MS or GC–MS/MS that are complicated, costly and time consuming rather than a simple HPLC–UV method. The method was successfully applied for pharmacokinetic study of lacidipine.
Keywords: Simple Method; High performance liquid chromatography; Validation; Lacidipine; Pharmacokinetic study.

Introduction

Lacidipine (1) is a calcium channel blocker drug. Lacidipine is a highly vascular selective newer dihydropyridines suitable for once daily administration. It is claimed to attain higher concentration in Vascular smooth muscle membrane; approved only for use as antihypertensive. Chemical name of Lacidipine is (E)-4-[2-[3-(1,1-Dimethylethoxy)-3-oxo-1-propenyl] phenyl]-1,4-dihydro-2,6-dimethyl-3,5-pyridine dicarboxylic acid diethyl ester. Only limited methods have been described in the literature for the determination of lacidipine by HPLC and LC-MS-MS (2-9). No single method was reported using HPLC (UV) detection. In the present study, a more sensitive, and precise (HPLC) method was to be developed and applied to the pharmacokinetic study.

Experimental

Materials and reagents

Acetonitrile HPLC grade was procured from Merck KGaA, Germany. Ammonium acetate AR grade were procured from SYSTEMRM, Selangor, Malaysia. Water HPLC grade was obtained from a Milli-QRO water purification system. Reference standards of Lacidipine and Felodipine were obtained from Cipla Health Care, Ahmadabad, India as a gift sample.

Equipment

HPLC chromatographic separation was performed on a Shimadzu liquid chromatographic system equipped with a LC-20AD solvent delivery system (pump), SPD-20A photo diode array detector, and SIL-20ACHT injector with 50µL loop volume. LC

solution version 1.25 was applied for data collecting and processing (Shimadzu, Japan).

Preparation of the calibration standards and quality control (QC) samples

The stock solutions of lacidipine and felodipine were prepared in water and acetonitrile mixture 1:1 at a concentration of 1.0 mg/mL each. Lacidipine working solution was used to prepare the spiking stock solutions for construction of six-point calibration curve (25.0 -150.0 ng/mL) and QC samples at three different levels (50.0, 100.0, 150.0 ng/mL). All the stock solutions were refrigerated (2-8° C) when not in use. Calibration standards and QC samples were prepared in bulk by spiking 25.0 µL of respective spiking stock solutions. These were stored at -70° C until analysis.

Sample preparation for analysis

Calibration standards, validation QC samples and healthy volunteer plasma samples were prepared by adding 0.5 mL plasma to 2.0mL centrifuge tube and added 0.5 ml (10 µg/mL) of internal standard and 0.5 ml of precipitating agent (10% v/v trichloroacetic acid) vortexed for 2 min. The resulting solution was centrifuged at 4000 rpm for 7 min. The supernatant layer was separated and estimated by HPLC.

Chromatographic conditions

The samples were chromatographed on a C_{18} (250 mm x 4.6 mm i.d., 5µ) column with a flow rate of 1.0 mL/min. The mobile phase used was methanol – ammonium acetate (70:30 % v/v). Ammonium acetate used was 50mM solution in water with pH being adjusted to 4.0 with orthophosphoric acid solution. The injection volume was 20.0 µL. The UV-visible detector was set at 320 nm.

Validation

The method has been validated (10) for selectivity, sensitivity, recovery, linearity, precision, accuracy and stability.

Selectivity

The selectivity of the method was evaluated by comparing the chromatograms obtained from the samples containing lacidipine and the internal standard with those obtained from blank samples.

Sensitivity

Sensitivity was determined in terms of LLOQ (Lower Limit of Quantification) where the response of LLOQ was at least five times greater than the response of interference in blank matrix at the retention time or mass transitions of the analyte.

Linearity

For linearity, different concentrations of standard solutions were prepared to contain 25.0 ng/mL to 150.0 ng/mL of lacidipine containing 5.0 µL of internal standard (50.0 µg/mL felodipine). These solutions were analysed and the peak areas and response factors were calculated. The calibration curve was plotted using response factor vs concentration of the standard solutions. Standard curve fitting was determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighing and statistical tests for goodness of fit.

Precision and Accuracy

The precision of the method was determined by intraday precision and interday precision. The intra-assay precision and accuracy was calculated for five replicates at each Lower Limit of Quantification (LLOQ), Low Quality Control (LQC), Middle Quality Control (MQC) and High Quality Control (HQC) levels, each on the same analytical run, and inter-assay precision and accuracy was calculated after repeated analysis in three different analytical runs.

Stability Studies

Stability study was carried out. Room temperature stock solution stability, refrigerated stock solution stability, freeze thaw stability, short term stability and long term stability were determined. Room temperature stock solution stability was carried out at 0, 3 and 8 hours by injecting four replicates of prepared stock dilutions of lacidipine equivalent to middle quality control sample concentration and the stock dilution of internal standard equivalent to the working concentration. Comparison of the mean area response of lacidipine and internal standard at 3 and 8 hours was carried out against the zero hour value. Refrigerated stock solution stability was determined at 7, 14 and 27 days by injecting four replicates of prepared stock dilutions of the analyte equivalent to the middle quality control sample concentration and the stock dilution of internal standard equivalent to the working concentration. The stability studies of plasma samples spiked with lacidipine were subjected to three freeze - thaw cycles, short term stability at room temperature for 3 h and long term stability at -70^{o} C over four weeks. In addition, stability of standard solutions was performed at room temperature over 6 h and after freezing for four weeks. The stability of triplicate spiked human plasma samples following three freeze thaw cycles was analysed. The mean concentrations of the stability samples were compared to the

theoretical concentrations. The stability of triplicate short term samples spiked with lacidipine was investigated at room temperature for 1.00 to 3.00 h before extraction. The plasma samples for long term stability were stored in the freezer at -70^0 C until the time of analysis.

Results and discussion

Selectivity

No interfering endogenous compound (Fig. 1) peak was observed at the retention time of analyte. Under chromatographic conditions, the retention times of lacidipine and felodipine were 9.62 min and 6.15 min respectively. Representative chromatograms of Lower Limit of Quantitation (LLOQ) and one study sample containing lacidipine is shown in Fig. 2 respectively.

Sensitivity (Lower limit of quantitation)

The sensitivity of the experiment was carried out at LLOQ level.

Linearity

The calibration curves were linear over the range of 25.0-150.0 ng/mL. The correlation coefficient was > 0.999. Calibration curve data of lacidipine result shown in Table 1 and Fig. 3.

Precision and Accuracy

Both intra-day and inter-day accuracy and precision of the method were determined by

Analysis of the control rat plasma spiked with lacidipine at LLOQ, LQC, MQC and HQC. All QCs concentration was calculated using the calibration curve. The accuracy and precision of the method were described as a percentage bias and the percentage relative standard deviation; the results are given in Table 2.

Fig: 1 Typical Chromatogram of Blank Plasma

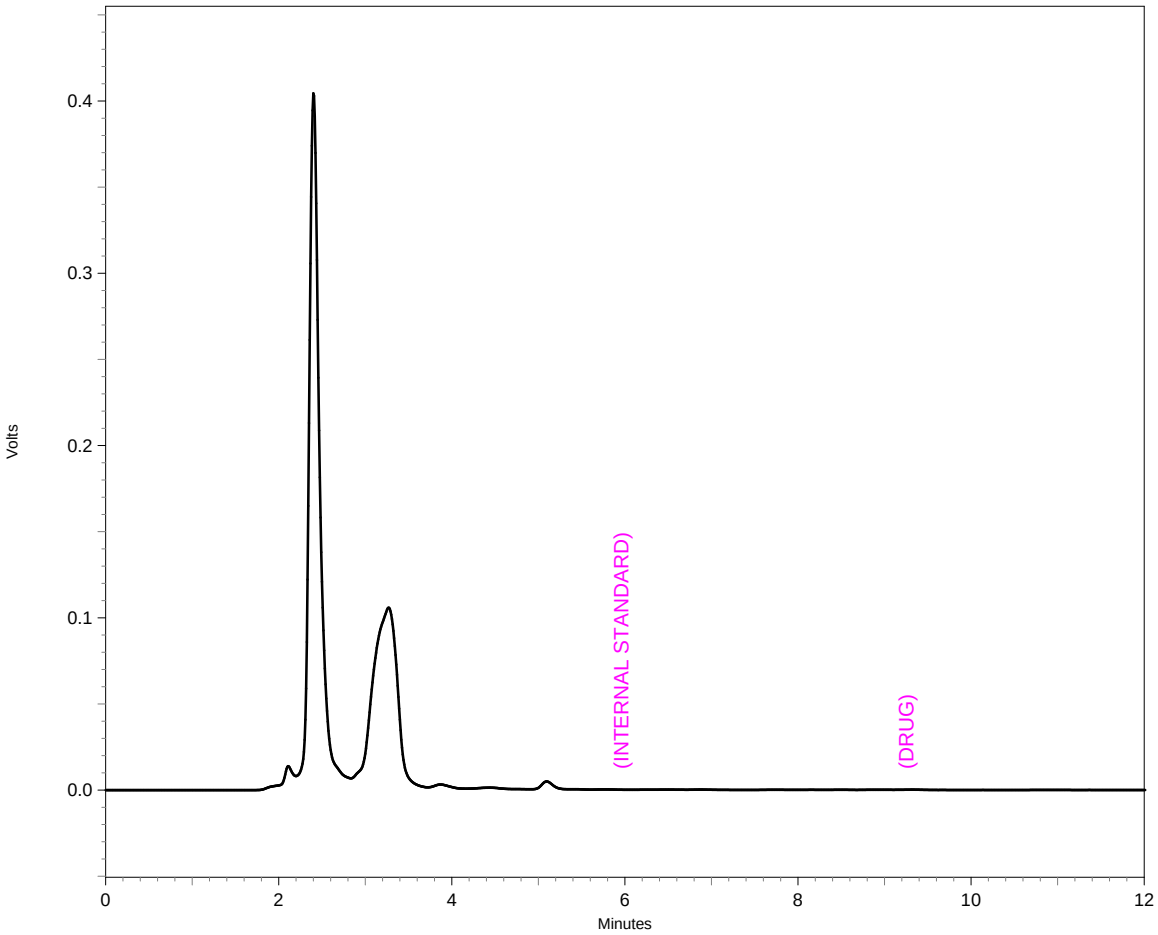
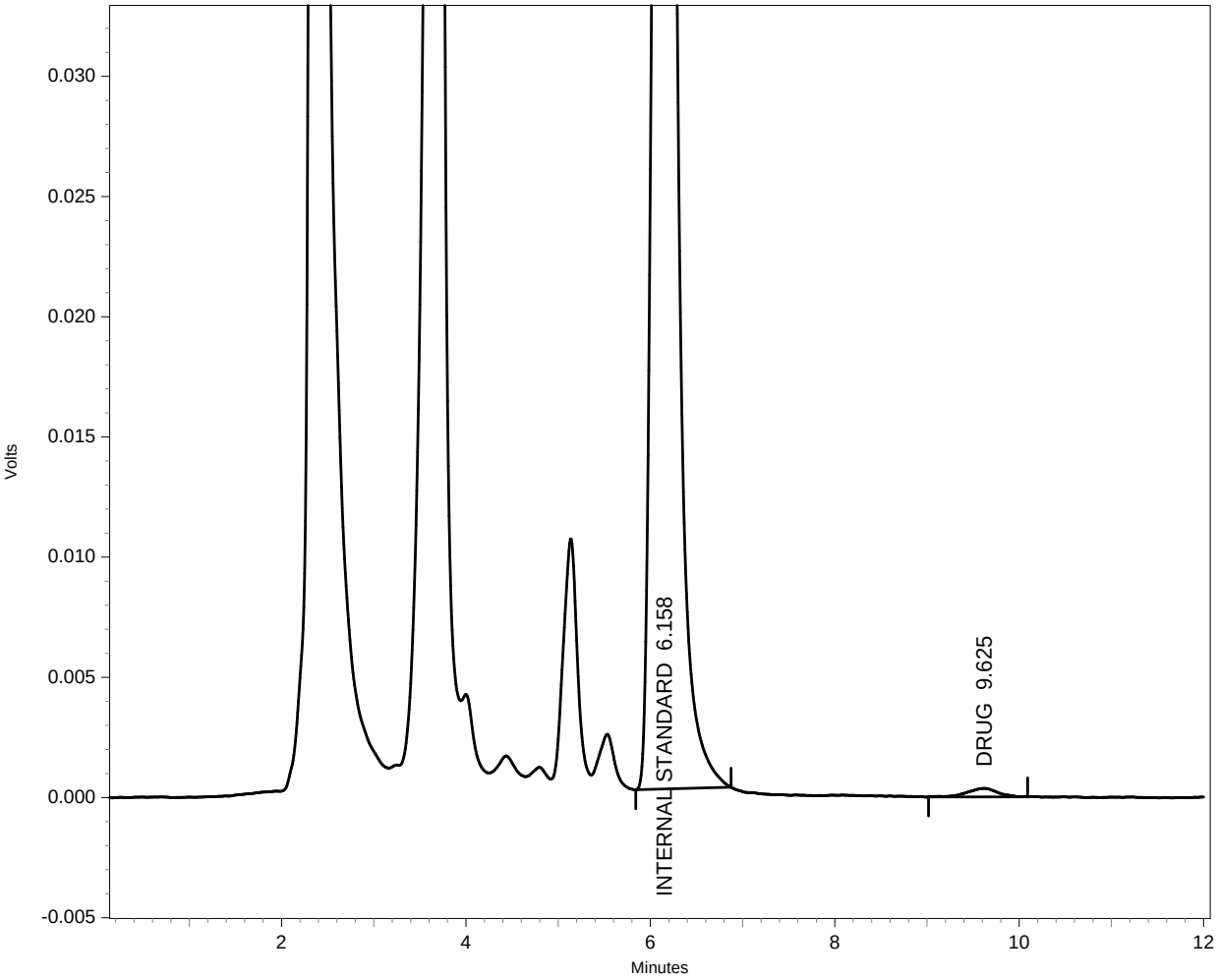


Fig: 2 Typical Chromatogram of Lacidipine Sample



Stability

Analysis of the stock solution was performed at 150.0 ng/mL. After storage for 15 days at 2-8 °C and at room temperature for 6h, more than 98 % of lacidipine remained unchanged, based on peak areas in comparison with freshly prepared solution of lacidipine. This suggests that the lacidipine in standard solution is stable for at least 15 days when stored at 2-8 °C and for 6h at room temperature.

Bench top stability of lacidipine in plasma was investigated at LQC and HQC levels. This revealed that the lacidipine in plasma was stable for at least 6 h at room temperature. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with lacidipine at LQC and HQC level did not affect the stability of lacidipine Long term stability of the lacidipine in plasma at -70 °C was also performed after 30 days of storage at LQC, HQC levels. The results of the stability studies are shown in Table 3. The average long term stability was 95.75%. The above results indicated that the lacidipine was stable in the studied conditions [Table 3].

Table 1. Inter-run accuracy and precision of plasma calibration standards for Lacidipine

Standard concentration (ng/mL)	Average calculated Concentration (ng/mL)	SD
25	24.81	5.75
50	149.05	2016
75	74.08	3.15
100	99.91	1.89
125	124.56	1.75
150	149.91	0.98

SD=Standard deviation

Table 2. Intraday and Interday accuracy and precision of Lacidipine in plasma

Standard concentration (ng/mL)	Average calculated concentration (ng/mL)	SD
Inter-day (n=3)		
50	48.96	0.85
100	99.36	0.49
150	149.53	0.58
Intra-day (n=3)		
50	49.06	0.79
100	99.40	0.51
150	149.73	0.50

Fig: 3 Calibration Curve of Lacidipine

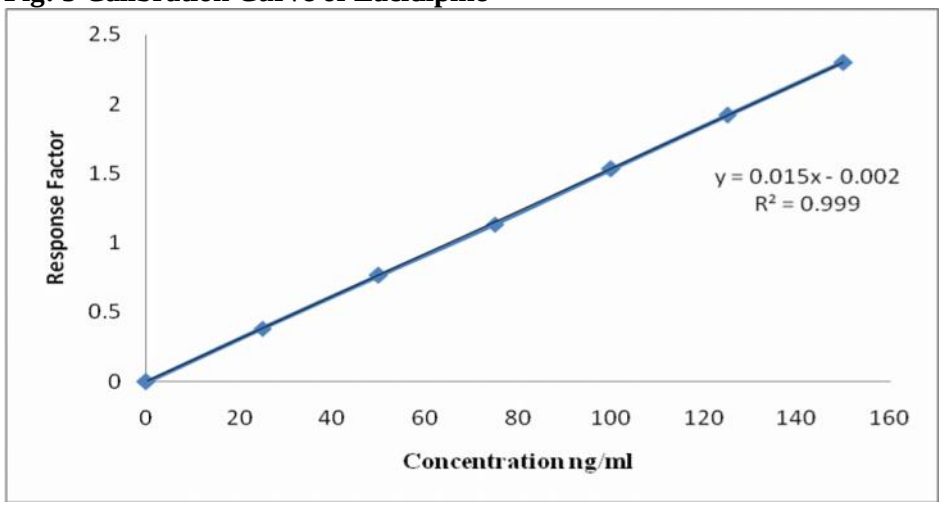


Table 3. Stability Study of Lacidipine

Standard concentration (ng/mL)	Average calculated concentration (ng/mL)	SD
Bench top (n=5)		
50	48.68	0.66
150	149.25	1.06
Freeze thaw Stability (n=5)		
50	49.14	0.79
150	149.52	0.94
Long term Stability (n=5)		
50	48.33	0.45
150	148.93	0.91

Conclusion

A simple and sensitive method for the determination of lacidipine in plasma by HPLC was developed and validated. The method consisted of sample preparation by Protein precipitating method, followed by chromatographic separation and UV detection. No interfering peaks were observed at the elution times of lacidipine and IS. Adequate specificity, precision and accuracy of the proposed method were demonstrated over the concentration range of 25.0-150.0 ng/mL. The method was accurate, reproducible, specific and applicable to the evaluation of pharmacokinetic profiles of lacidipine in rats. The developed HPLC method was found to be suitable for the analysis of lacidipine in human plasma. This method was applied for the pharmacokinetic study of lacidipine.

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